THE UNIVERSITY
of EDINBURGH

The Role of Hepatic 11β-Hydroxysteroid Dehydrogenase Type 1 (11β-HSD1) in Cholesterol Homeostasis

Kajal Manwani

Presented for the Degree of Doctor of Philosophy

The University of Edinburgh

2015

Declaration

I hereby declare that this thesis was written by me and that the data presented in this thesis represent my own work, with exceptions listed below, performed at The University of Edinburgh under the supervision of Professors Karen Chapman and Jonathan Seckl.

Plasma glucose and insulin measurements were performed by Dr. Sophie Turban.

Collagen staining and adipocyte sizes in picrosirius red stained subcutaneous fat samples were recorded by Mr. Oliver Brown.

I also declare that this work has not been submitted for any other degree or professional qualification.

Kajal Manwani
Edinburgh, 2015

Abstract

Chronic glucocorticoid (GC) excess (Cushing's syndrome, pharmacotherapy) causes metabolic and cardiovascular disease. This might be predicted from the known metabolic (dyslipidaemia, insulin resistance/hyperglycaemia) and hypertensive effects of chronically elevated GC levels. Intracellular GC levels within target tissues are controlled by 11β-hydroxysteroid dehydrogenases. 11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1, encoded by *Hsd11b1*) is an enzyme that, in intact cells and *in vivo*, converts inert GCs (cortisone in humans, and 11-dehydrocorticosterone in mice and rats) into their active forms (cortisol and corticosterone, respectively). Consequently, 11β-HSD1 amplifies intracellular GC levels. Additionally, 11β-HSD1 is also involved in the metabolism of 7-oxysterols; it catalyses the reduction of 7-ketocholesterol (7-KC) to 7β-hydroxycholesterol (7β-HC). 7-KC may inhibit cholesterol biosynthesis through its ability to inhibit cleavage/processing of sterol regulatory element binding protein-2 (SREBP-2), the key regulator of cholesterol synthesis. Alteration of cholesterol homeostasis is a major risk factor for cardiovascular disease. Improvement of metabolic syndrome and attenuation of atherosclerosis are observed in susceptible rodent models with 11β-HSD1 deficiency or inhibition. Conversely, pilot data showed decreased levels of 7-KC as well as increased levels of cleaved SREBP-2 protein (the transcriptionally active form) in liver of mice with hepatic 11β-HSD1 overexpression (LOE mice), suggesting increased cholesterol biosynthesis. It was hypothesised that hepatic 11β-HSD1 promotes cholesterol biosynthesis through hepatic induction of SREBP-2 target genes in the cholesterol biosynthetic pathway.

The hypothesis was tested in adult, male LOE and wild-type C57BL/6 mice. In mice fed a standard chow diet, hepatic levels of mRNA encoding hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase and HMG-CoA synthase, SREBP-2 targets in the cholesterol biosynthetic pathway, did not differ between genotypes. Compared to chow, a cholesterol-rich 'Western' diet (WD) decreased hepatic levels of mRNA encoding SREBP-2, HMG-CoA reductase and HMG-CoA synthase in wild-type as well as in LOE mice. These data imply that LOE mice show a normal physiological response with respect to cholesterol synthesis when challenged with cholesterol-rich

diet, and, contrary to the hypothesis, liver 11β-HSD1 does *not* increase cholesterol biosynthesis via elevated expression of mRNAs encoding hepatic cholesterol biosynthetic enzymes.

The liver X receptors (LXR) are well-known as sensors of oxysterols and regulators of genes involved in processes that decrease total body cholesterol levels *i.e*. reverse cholesterol transport and cholesterol excretion into bile. Cholesterol is the precursor to oxysterol LXR ligands. It was predicted that liver overexpression of 11β-HSD1 leads to activation of LXRα (the isoform with dominant roles in reverse cholesterol transport and whole-body cholesterol homeostasis) and its downstream targets involved in cholesterol efflux and excretion, in response to increased intracellular cholesterol levels. Indeed, levels of *Lxrα* mRNA were increased in livers of WD-fed LOE mice compared to wild-type mice on the same diet. There was no evidence for increased cholesterol clearance through bile acid synthesis in LOE mice as indicated by unchanged levels of hepatic *Cyp7a1* mRNA between LOE and wild-type mice. However, consistent with being direct targets of LXRα, increased *Abcg5* and *Abcg8* mRNA levels were observed in livers of WD-fed LOE mice compared to WD-fed wild-type mice. These results corroborate findings in chow-fed LOE mice. Moreover, these data suggest that LOE mice 'sense' intracellular cholesterol excess and respond to it by increasing cholesterol efflux into the biliary lumen for excretion, thereby supporting a role for hepatic 11β-HSD1 in promoting biliary cholesterol secretion.

To assess the effect(s) of hepatic 11β-HSD1 deficiency on cholesterol homeostasis as well as evaluate the importance of liver 11β-HSD1 in metabolic syndrome, liver-specific 11β-HSD1 knockout (LKO) mice were generated by crossing "floxed" *Hsd11b1* mice with *Alb-Cre* transgenic mice in which Cre expression is restricted to hepatocytes. In liver of LKO mice, 11β-HSD1 mRNA, protein and enzyme activity were reduced by >80%, with no differences in 11β-HSD1 protein levels in kidney, adipose tissue or muscle between LKO and floxed *Hsd11b1* littermate controls. These results indicate liver-specificity of *Hsd11b1* knockdown in these mice. Body weight and weights of liver, adipose tissue, adrenal, muscle, kidney and brain were unaltered by liver-specific 11β-HSD1 deficiency on a standard chow diet. These mice were subject to a 14-week high fat (HF) diet, which typically causes metabolic syndrome in

control but not globally 11β-HSD1 deficient mice. In HF-fed LKO mice, weights of the subcutaneous and epididymal fat depots were decreased compared to HF-fed control mice, resulting in an overall decrease in total white adipose tissue weight. Although no differences were observed in subcutaneous adipocyte hypertrophy between HF-fed LKO and control mice in a small number of samples tested, the above finding suggests that liver 11β-HSD1 influences adiposity and that liver-specific deficiency of 11β-HSD1 may reduce diet-induced adiposity.

In terms of cholesterol homeostasis, no differences were observed in hepatic levels of mRNAs encoding cholesterol biosynthetic enzymes as well as those encoding enzymes/transporters for cholesterol catabolism/excretion between LKO and control mice, on either chow or HF diet.

In summary, these data do *not* support a role for hepatic 11β-HSD1 in cholesterol synthesis. However, my evidence suggests that *increased* hepatic 11β-HSD1 promotes hepatobiliary cholesterol secretion. Finally, knockdown of liver 11β-HSD1, combined with HF feeding, reduces adiposity, suggesting that hepatic 11β-HSD1 may play a key role in adipose tissue lipogenesis/lipolysis and/or lipid storage, and that liver-specific 11β-HSD1 inhibition (or deficiency) may be advantageous in diet-induced obesity. Data presented in this thesis contribute to the understanding of the role of hepatic 11β-HSD1 in cholesterol homeostasis and metabolic syndrome.

Lay Summary

Prolonged glucocorticoid excess causes metabolic syndrome and cardiovascular disease. This might be predicted from the known metabolic (dyslipidaemia, insulin resistance) and hypertensive effects of chronically elevated glucocorticoid levels. In cells of target tissues, active glucocorticoid levels are controlled by an enzyme known as 11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1).

It has been long known that 11β-HSD1, in intact cells, coverts inactive glucocorticoids into their active forms, thereby amplifying intracellular glucocorticoid levels. Improvement of metabolic syndrome and attenuation of atherosclerotic heart disease are observed in susceptible rodent models with 11β-HSD1 deficiency or inhibition. Recent studies have revealed that 11β-HSD1 is also involved in the metabolism of 7-oxysterols, which are oxygenated cholesterol derivatives. Alteration of cholesterol homeostasis is a major risk factor for cardiovascular disease. It was hypothesised that hepatic 11β-HSD1 plays a role in cholesterol homeostasis. I investigated this hypothesis using mouse models of liver 11β-HSD1 over-expression and liver-specific 11β-HSD1 deficiency. My data suggest that increased liver 11β-HSD1 promotes hepatobiliary cholesterol secretion, both on control and cholesterol-rich diets. Deficiency of liver 11β-HSD1, combined with high fat feeding, resulted in reduced white fat weight, suggesting that hepatic 11β-HSD1 deficiency or inhibition may be advantageous in diet-induced adiposity/obesity.

The implications of this work are broad due to the current obesity and heart disease epidemics in humans. Diet-induced obesity studies in rodents are fundamental to unravel the complex mechanisms underlying this multifaceted disease, ultimately resulting in the identification of new preventive and therapeutic strategies.

Acknowledgements

Undertaking this PhD has truly been a life-changing experience for me and one of the joys of completion is to look back at the journey and remember all those who supported me along this fulfilling path. I would like to sincerely thank each person who has been important, in one way or another, to the successful realisation of this thesis.

Firstly, I would like to thank my supervisor Professor Karen Chapman, for her patience and guidance throughout my PhD studies. It has been an honour, indeed, to be one of her many PhD students. She has taught me, both, consciously and unconsciously, how good experimental science is done, and has always encouraged me to really think about important questions and the wider implications of experimental results. The joy and enthusiasm she has for her work have been a source of inspiration and motivation for me. I am also grateful for the excellent example she has provided as a successful female scientist, professor and working mother, always reminding me that "where there's a will, there's a way!"

I would also like to give a heartfelt, special thanks to Professor Jonathan Seckl, for giving me the opportunity to work on this very interesting project, as well as for being a motivating, encouraging and enlightening supervisor. I am particularly grateful to him for his intellectually stimulating discussions and questions about the "big picture" of the study.

Thirdly, I am extremely thankful to Dr. Ruth Andrew, for being a member of my thesis committee and providing excellent academic support and input.

This PhD would not have been possible without the numerous lab members who had the expertise to teach me different techniques and the patience to answer my (sometimes) endless (and often, quite silly!) questions. Tak Yung Man kindly and patiently took me through all the techniques related to animal work, from keeping good breeding records to carrying out clean mouse dissections. She has the ability to turn work into play and there was never a dull moment with her, even under extreme

pressure of challenging techniques and tight deadlines. I also have great regard for Val Kelly and June Noble, whom I have bothered on frequent occasions, often on very short notice, for solution recipes and various protocols, but in all instances I was received with warm smiles, and presented with protocols in essential lab techniques as well as bonus time-saving tips/tricks! I have also learnt a lot just by watching them in lab, most valuable lesson of which is the importance of keeping calm when things do not go as planned. Warm thanks go to Dr. Tijana Mitic who helped me to get preliminary data for this study, and also eased the process of settling into the lab. My special thanks are also due to Scott Noble and the entire BRF staff, both at the George Square campus as well as the Little France campus.

The members of Chapman and Holmes groups have enormously contributed to my personal and professional time at Edinburgh University. These groups have been a source of friendships as well as good advice. Many thanks go, in particular, to James, Mei, Monica, Manu, Eva, and Rachel, with whom I have gone through long hours in lab. We have also had the opportunity to share endless discussions on practically anything from science to food, conferences to holidays, careers to hobbies. It is, indeed, a pleasure to convey my gratitude to all of them in my humble acknowledgements.

I gratefully acknowledge the funding sources that made my PhD research possible. The College of Medicine and Veterinary Medicine, University of Edinburgh, as well as the competitive SORSAS award, funded my PhD work. It is also a pleasure to extend my thanks to the Cardiovascular Sciences department at the Queen's Medical Research Institute who provided the support and equipment I have needed to produce and complete my thesis.

My time in Edinburgh was made enjoyable due to the many great friends that became a part of my life. I am grateful for time spent with friends and family who visited for the many adventurous and memorable trips all over the UK. Special thanks go to Supreya, Avanish, Kiran, as well as the Prakash sisters (Gayathri & Kasthuri), for their hospitality, loving care, and understanding, as I rattled on about my PhD work.
I will be forever thankful to my undergraduate study advisor, Dr. Iain Allison, who first encouraged me to "go for a PhD". He has been a great mentor and friend,

providing advice, references, stories and laughs, through the years. Another mentor, without whom this journey would be incomplete, is Dr. Mohandas Hegde. He has been an anchor, through and through; his guidance and support always came when needed most. I look up to him because he is spiritually guided and always works from his heart. I hope I have inculcated some of these qualities in me.

Some of my cousins and high-school friends have been constant pillars of strength for me. I wish to affectionately thank Rinku, Poonam, AP (my sister from another mother!), Ajay and Ekta. Despite the great geographical distances, they have supported me during difficult times, always reminding me of my past and present accomplishments. Their pride in me helped me through times when I did not have faith in myself, and for this I am ever grateful to them.

Most importantly, I would not be here without my family! I owe my loving thanks to my parents for their endless support and prayers. My father, "Papa", is the person who first showed me the joy of intellectual pursuit when I was a child, by taking me to various libraries, book fairs and bookshops during his free time. I now fondly look back to those days that triggered my love for reading and thirst for knowledge. My darling mother, "Mommy", is the one who sincerely brought me up with her caring, gentle and unconditional love, and without her moral support, surviving the final stages of my PhD study would be unimaginable. Puneet, thanks for being the most loving brother and helping me take life a little less seriously, with a touch of humour. My grandmother, "Tu tu mama", has filled my heart with light and hope through her kindness and love, and without her upbringing, I would not be the person I am today.

The best outcome from these last few years in Edinburgh is finding my best friend, soul mate, and husband, Mayank Gupta. I thank him from the bottom of my heart for sticking by my side, through thick and thin. I am glad that, through these years, we have both learned a lot about life and strengthened our commitment to each other and to live life to the fullest. I would also like to extend my heart-felt thanks to my new family, Mummy, Daddy, Priyank and Shubhi, for lovingly welcoming me into the family, and providing immense support to take me through the challenging last stages of my PhD.

Finally, no words will suffice to convey my gratitude to my spiritual guide, Shri Parthasarathi Rajagopalachari, without whom my life would not have a meaningful purpose. Hence, I lovingly dedicate this thesis to Him.

"Nothing is, as easy as it looks, nor as difficult as it seems to be. Nothing!"

- Shri Ram Chandra, Shahjahanpur, India.

To my Spiritual Guide, Shri Parthasarathi Rajagopalachari

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ABC	ATP-binding cassette
ABCA1	ABC transporter A1
ABCG1	ABC transporter G1
ABCG5	ABC transporter G5
ABCG8	ABC transporter G8
ACTB	Actin, beta
ANOVA	Analysis of variance
aP2	Adipocyte fatty acid binding protein
ApoA1	Apo lipoprotein A-1
ApoE	Apo lipoprotein E
ATP	Adenosine triphosphate
AUC	Area under curve
bp	Base pair
BSA	Bovine serum albumin
C	Chow
CA	Cholic acid
CDCA	Chenodeoxycholic acid
cDNA	Complementary deoxyribonucleic acid
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CYP	Cytochrome P450
CYP7A1	Cytochrome P450 7A1 / cholesterol 7 alpha-hydroxylase
CYP7B1	Cytochrome P450 7B1 / 25-hydroxycholesterol 7-alpha-hydroxylase
CYP27A1	Cytochrome P450 27A1 / sterol 27-hydroxylase
Del	Global 11β-HSD1 knockout mice
DEPC	Diethylpyrocarbonate
11-DHC	11-Dehydrocorticosterone
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FFA	Free fatty acid
GC	Glucocorticoid
GR	Glucocorticoid receptor
GTT	Glucose tolerance test
h	Hour
H6PDH	Hexose-6-phosphate dehydrogenase
HDL	High-density lipoprotein
HF	High fat
HMGCR	Hydroxymethylglutaryl coenzyme A reductase
HMGCS	Hydroxymethylglutaryl coenzyme A synthase
HPA axis	Hypothalamus-pituitary-adrenal axis
HPLC	High-performance liquid chromatography
HPRT	Hypoxanthine phosphoribosyltransferase
HSD	Hydroxysteroid dehydrogenase
Hsd11b1$^{flox/flox}$	"Floxed" *Hsd11b1* mice
HSD1 KO	11β-HSD1 deficient (knockout) mice
11β-HSD1	11β-Hydroxysteroid dehydrogenase type 1
11β-HSD2	11β-Hydroxysteroid dehydrogenase type 2
7β-HC	7β-Hydroxycholesterol
7α-HC	7α-Hydroxycholesterol
IDL	Intermediate-density lipoprotein
IgG	Immunoglobulin G
ITT	Insulin tolerance test
kb	Kilobase
7-KC	7-Ketocholesterol
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LKO	Liver-specific 11β-HSD1 knockout mice
LOE	Mice overexpressing 11β-HSD1 in the liver

LPL	Lipoprotein lipase
LXR	Liver X receptor
LXRα	Liver X receptor alpha
LXRβ	Liver X receptor beta
min	Minute
MOPS	3-(N-morpholino) propanesulfonic acid
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
NaOH	Sodium hydroxide
NAD/NADH	Nicotinamide adenine dinucleotide (oxidised/reduced)
NADP/NADPH	Nicotinamide adenine dinucleotide phosphate (oxidised/reduced)
NPC1L1	Niemann-Pick C1 like 1
O_2	Oxygen
PCR	Polymerase chain reaction
PIC	Protease inhibitor cocktail
qPCR	Quantitative PCR
RCT	Reverse cholesterol transport
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal RNA
RT	Room temperature
s	Second
SDR	Short-chain dehydrogenase/reductase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SR-B1	Scavenger receptor B1
SRE	Sterol response element
SREBP	Sterol response element binding protein
SREBP-1	Sterol response element binding protein 1
SREBP-2	Sterol response element binding protein 2
TBE	Tris boric acid EDTA
TBP	TATA binding protein

TG	Triglyceride
TICE	Transintestinal cholesterol efflux
TLC	Thin-layer chromatography
VLDL	Very low-density lipoprotein
v/v	Volume to volume ratio
WD	Western diet
WT	Wild-type
w/v	Weight to volume ratio

Chapter 1. Introduction

1. Introduction

Dyslipidaemia, as the name suggests, is an abnormality in the amount of lipids and lipoproteins in the blood; it is characterised by increased plasma levels of triglycerides, total cholesterol, and low-density lipoproteins, and decreased levels of high-density lipoprotein (Van Gaal *et al*., 2006). Dyslipidaemia is a component of the metabolic syndrome and is strongly associated with an increased risk of atherosclerosis, type 2 diabetes, obesity and fatty liver disease, all major causes of death and disability in many parts of the world (Paterson *et al*., 2004; Beaven and Tontonoz, 2006). In the above-mentioned conditions, excess cholesterol and/or altered plasma lipid profile influence whole-body cholesterol storage and removal. Steroid hormones such as glucocorticoids (GCs) can also contribute to the development of these diseases; chronic GC excess promotes dyslipidaemia and insulin resistance/hyperglycaemia, and is strongly associated with increased cardiovascular events (Walker, 2007; Hadoke *et al*., 2009). 11β-hydroxysteroid dehydrogenase (HSD) enzymes regulate intracellular GC levels by catalysing inter-conversion of active GCs and their inert forms. While 11β-HSD2 inactivates GCs, 11β-HSD1 typically regenerates active GCs in tissues where it is expressed, therefore amplifying intracellular GC levels (Seckl and Walker, 2001; Chapman and Seckl, 2008). Knockout of 11β-HSD1 in mice improves key cardiovascular risk factors associated with liver and adipose tissue function (Kotelevtsev *et al*., 1997; Morton *et al*., 2004), and pharmacological inhibition of 11β-HSD1 reduces atherosclerosis (Hermanowski-Vosatka *et al*., 2005). 11β-HSD1 also catalyses the reduction of oxysterols (Schweizer *et al*., 2004; Hult *et al*., 2004), which are believed to play a role in the regulation of cholesterol homeostasis. This thesis will explore the role of hepatic 11β-HSD1 in cholesterol homeostasis.

1.1. Glucocorticoids and Risk Factors for Cardiovascular Disease

Glucocorticoids are endogenous steroid hormones. The active forms are found as cortisol in humans and corticosterone in rats and mice. GCs have profound effects on physiological systems as they play a role in regulating the stress response (Sapolsky *et*

al., 2000), intermediary metabolism (McEwen *et al.*, 1997) as well as cognition (Setiawan *et al.,* 2007). GCs provide a "brake" on innate inflammatory mechanisms, and generally display anti-inflammatory and immunosuppressive actions (McEwen *et al.*, 1997; Sapolsky *et al*., 2000). Hence, they are widely prescribed to treat a number of chronic inflammatory conditions (including rheumatoid arthritis, asthma, and inflammatory bowel disease), as well as for immune suppression in organ transplantation patients.

At the cellular level, a great number of GC effects are mediated by the action of GCs on two types of intracellular receptor: glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). MRs have a high affinity for physiological GCs whereas GRs have a lower affinity for them, so are only occupied at higher concentrations of GCs (Funder, 1997; Reul *et al*., 2000). MR is expressed in a restricted range of tissues notably the distal nephron of the kidney, the colon and salivary glands, whereas GR is widely expressed and at higher levels than MR at most sites (reviewed by Chapman *et al*., 2013).

Under conditions of stress (such as starvation or exercise), GCs stimulate pathways to maintain blood glucose levels, including hepatic gluconeogenesis, protein breakdown (from muscle) and lipolysis (from adipose tissue) (Friedman *et al*., 1993; Slavin *et al*., 1994; Hanson and Reshef 1997; Buckingham, 2006; Vegiopoulous and Herzig, 2007). GCs also inhibit glucose uptake by muscle and fat during conditions of stress (Horner *et al*., 1987).

Although beneficial in adapting to short-term stress, chronic exposure to elevated GC levels has detrimental effects (Sapolsky *et al*., 1986; Dallman *et al.,* 2004). The metabolic and cardiovascular consequences are evident in Cushing's syndrome (caused by pituitary adenomas, ectopic adrenocorticotropic hormone-producing tumours or long term administration of synthetic GCs) that is characterised by central obesity, insulin resistance, hyperglycaemia, dyslipidaemia and hypertension, all major risk factors for cardiovascular disease (Bjorntorp and Rosmond, 1999; Walker *et al*., 2000). Apart from the density of glucocorticoid receptors, GC action on target tissues

is determined by intracellular metabolism by 11β-hydroxysteroid dehydrogenases, and therefore these enzymes are key to understanding here.

1.2. 11β-Hydroxysteroid Dehydrogenases

Short-chain alcohol dehydrogenases/reductases (SDRs) comprise a large family of enzymes that are evolutionarily conserved (Oppermann *et al*., 2003). This family includes mammalian enzymes such as 15-hydroxyprostaglandin dehydrogenase and 17β-hydroxysteroid dehydrogenase, as well as 11β-hydroxysteroid dehydrogenase. Even though only about 10-30% homology exists between the molecular sequences of the different dehydrogenases, the three-dimensional structures are highly similar (Filling *et al*., 2002). Most recognised SDR enzymes are the NADH or NADPH-dependent oxidoreductases that display distinct tissue expression patterns.

1.2.1. 11β-Hydroxysteroid Dehydrogenase Type 1 and Type 2

11β-Hydroxysteroid dehydrogenases (11β-HSD) are microsomal enzymes that come under the SDR class of enzymes (Edwards *et al*., 1988), and regulate intracellular GC levels by catalysing the inter-conversion of active GCs (cortisol in humans, corticosterone in rodents) and their inert 11-keto forms (cortisone in humans, 11-dehydrocorticosterone in rodents) (Monder and Shackleton, 1984; Seckl and Chapman, 1997). There are two isozymes of this enzyme; 11β-HSD type 1 (11βHSD1) and 11β-HSD type 2 (11β-HSD2), the products of distinct genes (Stewart and Krozowski, 1999). While 11β-HSD2 inactivates GCs, 11β-HSD1 is a reductase *in vivo*, regenerating active GCs from inactive forms (Figure 1.1), therefore locally amplifying GC levels available for activation of GRs within target cells (Seckl and Walker, 2001; Chapman and Seckl, 2008).

11β-HSD1 is expressed predominantly in GC target tissues including liver, adipose tissue, lung, and brain (Krozowski *et al*., 1990; Seckl and Chapman, 1997). On the other hand, 11β-HSD2 is more restricted, with expression in aldosterone target tissues such as kidney as well as in tissues of the developing embryo and placenta (Agarwal *et al*., 1990; Edwards, 1991; Walker and Edwards, 1991; Muneyyirci-Delale *et al*.,

1996; Seckl and Chapman, 1997; Gitau *et al*., 1998; Moore *et al*., 2000; Draper and Stewart, 2005). The enzyme 11β-HSD1 forms the focus of the work described in this thesis.

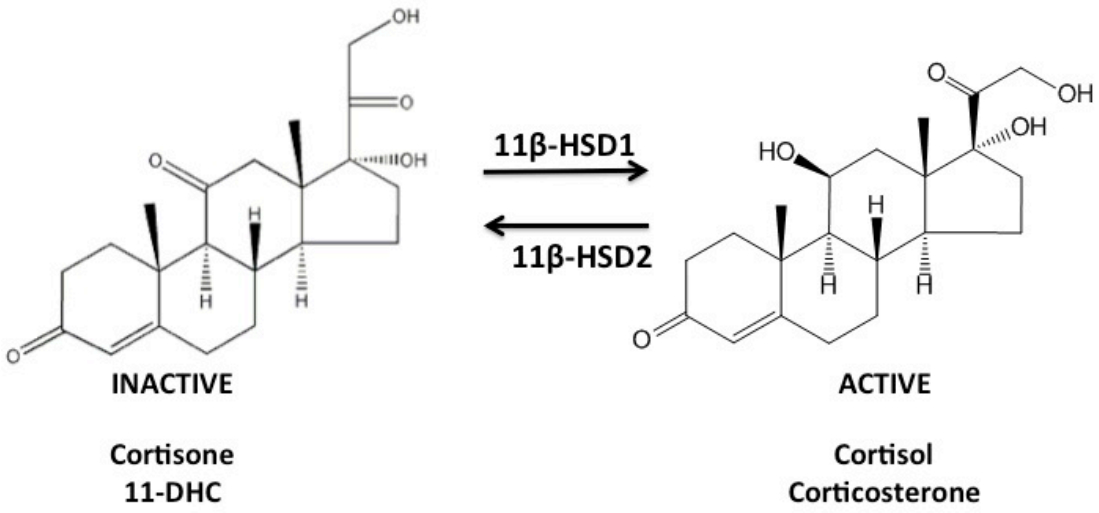


Figure 1.1: 11β-Hydroxysteroid dehydrogenases (11β-HSDs) catalyse the intracellular interconversion of 11-keto forms (cortisone, 11-dehydrocorticosterone) and 11-hydroxyl forms (cortisol, corticosterone) of glucocorticoids.

Arrows indicate the reaction direction *in vivo*, but 11β-HSD1 can act as a dehydrogenase in cell-free conditions (see text for details). The specific glucocorticoids depicted in this figure are cortisone and cortisol, which are found in humans. 11-DHC, 11-dehydrocorticosterone.

1.3. 11β-HSD1: Gene structure and Enzymology

The encoding gene, *HSD11B1*, is found near the end of the long arm of chromosome 1 in mice and humans, and chromosome 13 in rats (reviewed by Chapman *et al*., 2013). It comprises seven exons, with exons 2-7 encoding the full-length protein (reviewed by Chapman *et al*., 2013). There are three promoters, P1, P2 and P3, in the *HSD11B1* gene (Moisan *et al*., 1992; Bruley *et al*., 2006), with P2-initiated transcripts found in most tissues including liver, adipose and brain (Bruley *et al*., 2006; Staab *et al*., 2011) and P1-initiated transcripts predominating in mouse lung but hardly found in human lung (Staab *et al*., 2011). Transcription from the P3 promoter encodes a protein without 11β-HSD1 activity (Mercer *et al*., 1993) and its biological relevance is unclear (reviewed by Chapman *et al*., 2013).

The P2 promoter of *Hsd11b1* contains several binding sites for the CCAAT/enhancer binding protein (C/EBP) transcription factors, and in liver, transcription from P2 promoter is dependent on C/EBPα (Williams *et al*., 2000). In a variety of cell types such as adipocytes, fibroblasts and lung epithelial cells, C/EBPβ mediates 11β-HSD1 regulation in response to proinflammatory cytokines, GCs, diet and other regulators (Gout *et al*., 2006; Arai *et al*., 2007; Sai *et al*., 2008; Ignatova *et al*., 2009; Yang *et al*., 2009).

11β-HSD1 is a 34kDa transmembrane glycosylated protein located inside the inner leaflet of the endoplasmic reticulum (ER), in close association with hexose-6-phosphate dehydrogenase (H6PDH), which supplies the co-factor NADPH that in turn allows 11β-reduction (Atanasov *et al*., 2004; Banhegyi *et al*., 2004; Bujalska *et al*., 2005; Atanasov *et al*., 2008). In tissue homogenates, 11β-HSD1 is bidirectional, catalysing both reductase and dehydrogenase reactions, depending on co-factor (and substrate) availability (reviewed by Chapman *et al*., 2013).

11β-HSD1 has broad substrate specificity. Apart from catalysing GC interconversion, 11β-HSD1 also interconverts 7-ketocholesterol and 7β-hydroxycholesterol (Hult *et al*., 2004; Schweizer *et al*., 2004; also see 1.7 and 1.8). The 7-keto-sterol substrates competitively inhibit GC metabolism, and vice versa, with GC substrates also

inhibiting the 7-keto-sterol reduction (Nashev *et al*., 2007; Wamil *et al*., 2008; Balazs *et al*., 2009). The 11β-HSD1 reaction direction is affected by these 7-sterols when H6PDH is limiting; while 7-ketocholesterol represses, 7β-hydroxycholesterol enhances cellular GC action at GR, and by increasing supply of NADPH to 11β-HSD1 (through overexpression of H6PDH) this effect is reduced (Wamil *et al*., 2008). It has also been shown that human 11β-HSD1 converts the secondary bile acid 7-oxo-lithocholic acid to chenodeoxycholic acid (Odermatt *et al*. 2011), and that 11β-HSD1 plays a role in detoxification of xenobiotics (Maser and Oppermann, 1997).

1.4. 11β-HSD1: Insights from mouse models

1.4.1. Knockout and Inhibition of 11β-HSD1

In order to study the importance of 11β-HSD1 *in vivo* as well as determine therapeutic potential, 11β-HSD1 knockout (*Hsd11b1*$^{-/-}$) mice were generated (Kotelevtsev *et al*., 1997). Deficiency/knockout of 11β-HSD1 improves multiple metabolic syndrome parameters (see below) as well as protecting against age-related cognitive loss and skin thinning (Sandeep *et al*., 2004; Yau *et al*., 2011; Tiganescu *et al*., 2013). Amongst the above-mentioned effects of 11β-HSD1 deficiency, this thesis will mostly focus on the metabolic disease parameters.

11β-HSD1 deficient mice have reduced levels of plasma triglyceride (TG) and non-esterified fatty acids (NEFA) (Morton *et a*l., 2001). These mice also have raised high-density lipoprotein (HDL) cholesterol, with elevated liver mRNA and serum levels of apolipoprotein AI (ApoAI) (Morton *et al*., 2001; for further detail on HDL and other lipoproteins, refer to 1.5.3). Furthermore, 11β-HSD1 knockout mice resist hyperglycaemia on high fat (HF) diet (Kotelevtsev *et al*., 1997; Morton *et al*., 2004), exhibit enhanced insulin sensitivity in liver and adipose tissue (Morton *et al*., 2001; Morton *et al*., 2003; Morton *et al*., 2004), and have reduced "disadvantageous" visceral fat depot upon HF feeding (Morton *et al*., 2004). On a high cholesterol diet, 11β-HSD1 knockout mice have lower plasma cholesterol and a higher HDL to total cholesterol ratio (compared to wild-type mice on the same diet), factors which are all associated with a cardioprotective phenotype (Morton *et al*., 2004). Indeed, 11β-

HSD1 deficiency is also observed to be atheroprotective. In C57BL/6 mice with a targeted deletion of the *Apoe* gene (which encodes apolipoprotein E), hepatic uptake of circulating cholesterol is diminished and development of aortic atherosclerotic plaques is observed when these mice are fed an atherogenic "Western" diet (Zhang *et a*l., 1992; Plump *et al*., 1992). Crossing *Hsd11b1*$^{-/-}$ mice with *Apoe* knockout mice (*Apoe*$^{-/-}$) showed that deficiency of 11β-HSD1 results in a substantial reduction of atherosclerosis in the dyslipidaemic *Apoe*$^{-/-}$ mice (Kipari *et al*., 2013; Garcia *et al*., 2013). Over and above improved metabolic risk factors, recent evidence suggests direct atheroprotective effects of 11β-HSD1 deficiency/inhibition. 11β-HSD1 inhibition in *Apoe*$^{-/-}$ mice reduces inflammatory gene expression in the vasculature as a protective mechanism against atherosclerosis (Luo *et al*., 2013). Also, Kipari *et al*. (2013) showed that transplantation of 11β-HSD1-deficient bone marrow cells into irradiated *Apoe*$^{-/-}$ mice confers atheroprotection, and the mechanism may involve cholesterol accumulation in macrophages as 11β-HSD1-deficient macrophages showed enhanced cholesterol export (Kipari *et al*., 2013).

The above evidence, along with studies on the role of GCs in the pathogenesis of metabolic and cardiovascular disease, point to the suggestion that 11β-HSD1 would be a valuable therapeutic target for type 2 diabetes, obesity, dyslipidaemia and atherosclerosis (Walker, 2007; Wamil and Seckl, 2007; Chapman *et al*., 2013). Consistent with this, pharmacological inhibition of 11β-HSD1 improves insulin resistance, glucose, insulin and glucagon levels in mouse models of obesity and type 2 diabetes (Hermanowski-Vosatka *et al.*, 2005; Lloyd *et al*., 2009). Also, selective inhibition of 11β-HSD1 dramatically decreases circulating and intra-aortic cholesterol levels in atherosclerosis-prone *Apoe*$^{-/-}$ mice (Hermanowski-Vosatka *et al.*, 2005), and carbenoxolone (a non-selective 11β-HSD1 inhibitor) reduces atherosclerosis in susceptible *Ldlr*$^{-/-}$ mice (Nuotio-Antar *et al*., 2007). As in mice, inhibition of 11β-HSD1 in humans improves several metabolic disease risk factors. Phase II clinical trials using selective 11β-HSD1 inhibitor ICNB13739 in type 2 diabetes patients have shown reduction in levels of plasma glucose and glycated haemoglobin A1c, possibly via increased insulin sensitivity (Rosenstock *et al*., 2010). In hyperlipidaemic subjects, the inhibitor reduces plasma cholesterol and TG levels (Rosenstock *et al*., 2010). Another clinical trial showed that selective inhibition of 11β-HSD1 results in

modestly reduced haemoglobin A1c, low-density lipoprotein (LDL) cholesterol, blood pressure as well as body weight in patients with type 2 diabetes and metabolic syndrome (Feig *et al*., 2011).

1.4.2. Tissue-specific Overexpression of 11β-HSD1

11β-HSD1 activity is consistently found to be increased (two- to three-fold) in subcutaneous adipose tissue of obese men and women (Rask *et al*., 2001; Rask *et al*., 2002; Paulmyer-Lacroix *et al*., 2002; Sandeep *et al*., 2005; Goedecke *et al*., 2006; Paulsen *et al.*, 2007). This effect is accompanied by a reduction in hepatic 11β-HSD1 activity in simple obesity in the absence of type 2 diabetes (Rask *et al*., 2001), but liver 11β-HSD1 activity is unaltered if obesity is complicated by type 2 diabetes (Stimson *et al*., 2011). To investigate the pathogenic implications of increased adipose 11β-HSD1, aP2-HSD1 mice were generated, using the adipocyte fatty acid binding protein (aP2) promoter (Masuzaki *et al*., 2001). These mice overexpress 11β-HSD1 by two-to-three-fold selectively in adipose tissue, modelling obese humans and rodents. Contrary to *Hsd11b1*$^{-/-}$ mice, the aP2-HSD1 mice fully replicate metabolic syndrome with insulin resistance/diabetes (which is exacerbated by HF feeding), dyslipidaemia, hypertension, hyperphagia and visceral obesity (Masuzaki *et al*., 2001).

Overexpression of 11β-HSD1 in adipose tissue increases intra-adipose corticosterone levels but circulating GC levels are unaltered (Masuzaki *et al*., 2001). Also, in aP2-HSD1 mice, corticosterone delivery to the liver is increased (approximately three-fold) through spillover of adipose corticosterone into the portal vein (Masuzaki *et al*., 2001). The enzyme 11β-HSD1 is most highly expressed in liver (Agarwal *et al*., 1989; Jamieson *et al*., 1995), and it has been suggested that heterogeneity of hepatic 11β-HSD1 may be relevant to the development of fatty liver and insulin-resistant phenotype (without obesity) in humans such as that observed in myotonic dystrophy where dyslipidaemia and insulin resistance occur with increased hepatic 11β-reduction of cortisone to cortisol (Johansson *et al.*, 2001; Masuzaki *et al*., 2001). To dissect the role of elevated hepatic GCs, transgenic mice overexpressing 11β-HSD1 selectively in liver (under the hepatic transcriptional control of the human *APOE* gene

promoter) were generated (Paterson *et al*., 2004). These liver overexpressor (LOE) mice show two- to five-fold increase (depending on the line) in 11β-HSD1 activity selectively in the liver, and display mild insulin resistance, fatty liver and dyslipidaemia, without impairment of glucose tolerance, obesity or changes in adipose mass distribution (Paterson *et al*., 2004). A similar phenotype of insulin resistance and fatty liver, irrespective of obesity, has been observed in patients with non-alcoholic fatty liver disease (Marchesini *et al*., 2003) and is a possible early indicator of more widespread insulin resistance and the full metabolic syndrome (McGarry, 1992). Interestingly, in response to HF diet, the LOE mice reveal altered cholesterol lipoprotein profile and attenuated hepatic induction of regulators of cholesterol homeostasis including LXRα (Paterson *et al*., 2004).

Along with the above indirect evidence, there is increasing direct indication that 11β-HSD1 may play a role in cholesterol homeostasis as it is involved in the metabolism of 7-oxysterols. Before further discussing the potential role of 11β-HSD1 in cholesterol homeostasis through the metabolism of its alternative substrates, a background on cholesterol homeostasis is important.

1.5. Cholesterol Homeostasis

Initially, cholesterol was discovered in bile and gallstones by Poulletier de la Salle in 1769, and then Chevrul rediscovered it in 1815, naming it "cholesterine". Cholesterol was later found in blood (Dam, 1958). Cholesterol is the primary sterol synthesised by animals. Apart from being an essential structural component of animal cell membranes establishing proper membrane fluidity and permeability, cholesterol is a vital precursor for the biosynthesis of steroid hormones, bile acids and Vitamin D (Simons and Ikonen, 2000; Hsu *et al*., 2006; Norlin and Wikvall, 2007). Despite its importance, animal cells cannot tolerate excess cholesterol. Raised levels of blood cholesterol and intracellular cholesterol accumulation can result in pathological consequences such as atherosclerotic cardiovascular disease and may also contribute to early onset of Alzheimer's disease and renal dysfunction (Kees-Folts and Diamond, 1993; Guyton and Klemp, 1996; Abrass, 2004; Wellington, 2004). It is, therefore, not

surprising that mammals have developed complex mechanisms to tightly control whole-body cholesterol levels and maintain cholesterol homeostasis (Goldstein and Brown, 1990; Brown and Goldstein, 1999; Jessup and Kritharides, 2008; Goldstein and Brown, 2009).

1.5.1. Cholesterol Biosynthesis

The liver is the major site of cholesterol synthesis; in mammals, approximately 70% of cholesterol is synthesised in the liver (Spady and Dietschy, 1983). The other major organ of cholesterol synthesis is the intestine, which accounts for nearly 20% of cholesterol production (Berg *et al*., 2002).

The complex cholesterol molecule, which contains 27 carbons, is synthesised from acetyl CoA, a two-carbon substrate, in a series of approximately 30 enzymatic reactions (Goldstein and Brown, 1990; Kelley and Herman, 2001; Kovacs *et al*., 2002). The first stage in the cholesterol biosynthetic pathway is formation of isopentenyl pyrophosphate from acetyl CoA. This set of reactions occurs in the cytosol, starting with the formation of 3-hydroxy-3methylglutaryl CoA (HMG CoA), which is then reduced to mevalonate. Synthesis of mevalonate is the committed step in cholesterol formation and the enzyme catalysing this irreversible step, 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR), is an important control site in cholesterol synthesis. Mevalonate is converted into 3-isopentenyl pyrophosphate. Six molecules of isopentenyl pyrophosphate are condensed to form squalene, which cyclises into a tetracyclic product (lanosterol) that is eventually converted into cholesterol in a multistep process (Berg *et al*., 2002) (Figure 1.2).

With the exception of HMGCR, the first enzymes of cholesterol biosynthetic pathway are soluble proteins found in the ER membrane (Goldstein and Brown, 1990; Gaylor, 2002; Kovacs *et al*., 2002). Reactions generating mevalonate can also occur within the peroxisome, and the succeeding reactions that result in the formation of farnesyl-pyrophosphate are exclusively peroxisomal (Olivier and Krisans, 2000). The remaining biosynthetic reactions take place in the ER (Figure 1.2). Hence, cholesterol

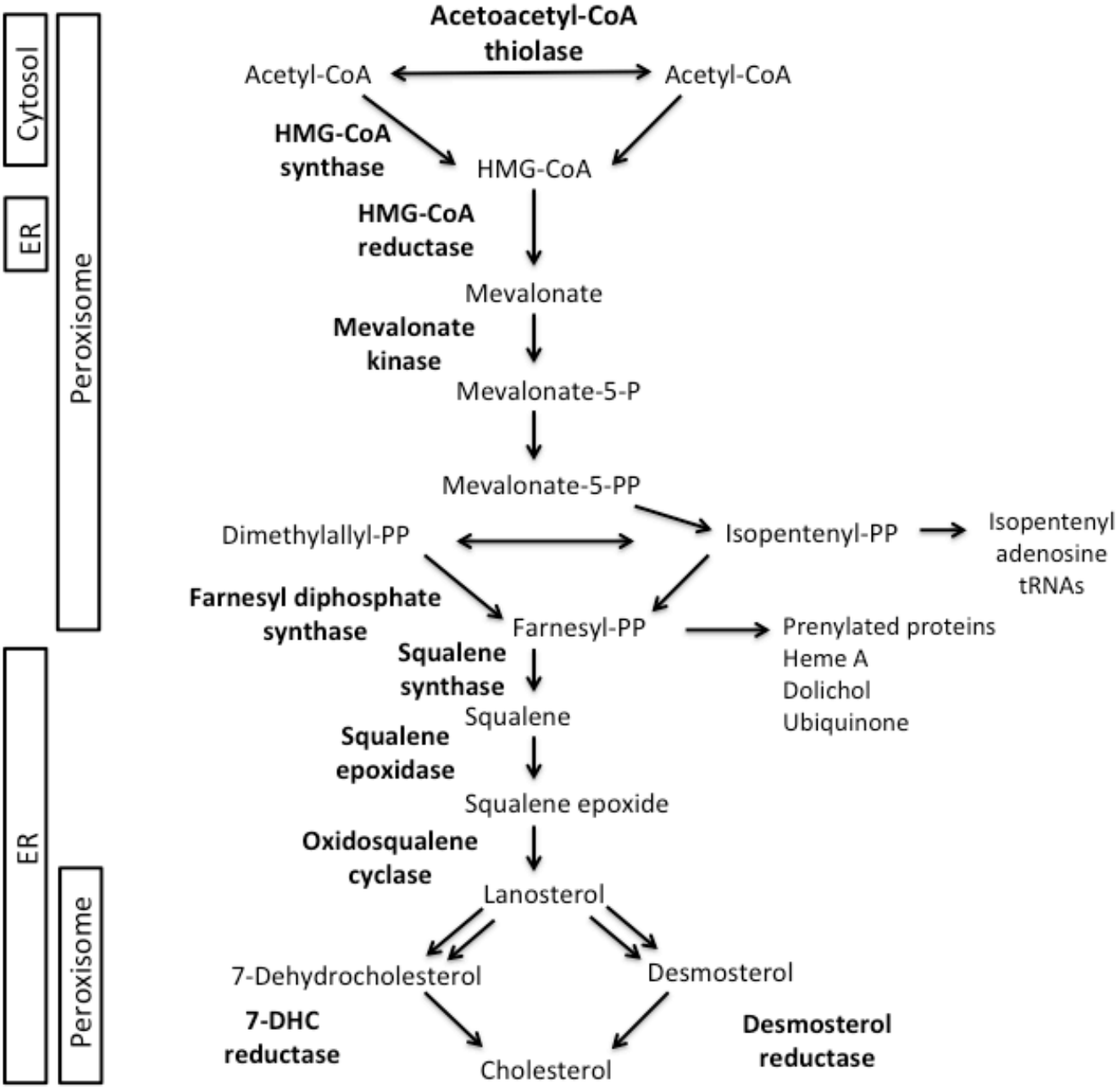


Figure 1.2: Cholesterol biosynthetic pathway.

A schematic representation of the cholesterol biosynthetic pathway, showing a number of cholesterol by-products and enzymes. Enzymes in the pathway are found in the cytosol, endoplasmic reticulum (ER) and peroxisomes. HMG, 3-hydroxy-3-methylglutaryl; DHC, dehydrocholesterol. Figure adapted from Liscum, 2002.

biosynthetic enzymes are compartmentalised in the cytosol, ER and/or peroxisome. The reason behind this is unclear but it has been suggested that this adds another level of complexity to the regulation of this important metabolic pathway (Olivier and Krisans, 2000; Liscum, 2002; Berg *et al*., 2002).

1.5.1.1. Regulation of Cholesterol Synthesis

Cholesterol can be obtained via the diet or it can be synthesised *de novo*. A remarkable example illustrating the regulatory power of cholesterol homeostasis is the case study of the 88-year old patient who ate around 25 eggs a day for approximately 15 years but still maintained normal serum cholesterol levels between 3.88 and 5.1mM (normal values in adults being <5.17mM) (Kern, 1991).

The rate of cholesterol biosynthesis depends on the availability of cholesterol in the diet. The cholesterol biosynthetic pathway is regulated by the sterol end product as well as non-sterol intermediates and other physiological factors, acting on several enzymes in the cholesterol biosynthetic pathway, though the focus has mostly been on HMGCR, the rate-limiting enzyme. Combined together, these regulatory factors can modify the amount of HMGCR over a 200-fold range. Whereas sterols regulate HMGCR gene transcription, non-sterols regulate its mRNA translation, and both sterols and non-sterols play a role in the stringent regulation of HMGCR protein degradation (Goldstein and Brown, 1990). Other physiological factors that influence cholesterol synthesis include diurnal rhythm, insulin and glucagon, GCs, oestrogen and bile acids, and these factors regulate HMGCR at transcriptional, translational as well as post-translational levels (Chambers and Ness, 1997; Ness and Chambers, 2000). Other enzymes in the cholesterol synthesis pathway that have been well studied include HMG-CoA synthase (HMGCS), farnesyl-PP synthase and squalene synthase (Brown and Goldstein, 1997; Edwards *et al*., 2000; Hampton 2000).

Each of the genes encoding the above-mentioned enzymes contains a sterol regulatory element (SRE) within their promoter regions that is recognised by a membrane-bound transcription factor, the sterol regulatory element binding protein (SREBP) (Brown and Goldstein, 1997). Cloning of SREBP cDNA (Yokoyama, 1993; Hua 1993)

revealed that there are two SREBP genes that produce three distinct proteins. SREBP-1a and -1c derive from one gene but from different promoters, whereas SREBP-2 is derived from a second gene (Brown and Goldstein, 1997). SREBPs not only regulate cholesterol synthesis but also the synthesis of fatty acids (Horton and Shimomura, 1999; Osborne, 2000). The functional roles were first deduced from studying transgenic mice overexpressing SREBP-1a, -1c, or -2 in liver (Shimano *et al*., 1996; Shimano *et al*., 1997; Horton *et al*., 1998). SREBP-1a is a strong activator of cholesterol and fatty acid synthesis and is likely to be important in cells that are rapidly dividing and need lipid for membrane production (Horton and Shimomura, 1999; Osborne, 2000). SREBP-1c is mainly in the liver, where it primarily activates genes of fatty acid synthesis, and it is important for maintaining basal transcription levels of fatty acid and cholesterol biosynthetic enzymes during fasting (Osborne, 2000; Horton *et al*., 2002). SREBP-2 selectively activates cholesterol biosynthetic genes and mainly responds when the liver's demand for cholesterol increases (Horton *et al*., 1998; Pai *et al*., 1998).

Sterol regulatory element binding proteins are cytosolic 68kDa proteins and belong to the basic helix-loop-helix leucine zipper (bHLH-Zip) family of transcription factors. However, unlike other bHLH-Zip transcription factors, SREBPs are 125kDa trans-membrane proteins located in the ER that undergo a two-step cleavage process to generate an N-terminal 68kDa fragment including the bHLH-Zip motif, which travels to the nucleus to activate transcription of genes involved in cholesterol and fatty acid synthesis. The C-terminal domain serves a regulatory function (Brown and Goldstein, 1997; Horton and Shimomura, 1999; Osborne, 2000). The sequential two-step cleavage of the full-length precursor SREBP is dependent on the cellular cholesterol content.

Three proteins required for the regulation of SREBP cleavage have been identified using somatic cell genetic approaches (Chang *et al*., 1997); SREBP cleavage-activating protein (SCAP), an escort protein, and two proteases, Site-1 protease (S1P) and Site-2 protease (S2P) (Chang *et al*., 1997; Goldstein *et al*., 2002). After synthesis in the ER, SREBPs complex with SCAP (Sakai and Rawson, 2001). SCAP is both a chaperone for SREBPs as well as a sensor of sterols (Brown *et al*., 2002). When cellular cholesterol levels are low, SCAP escorts SREBP from the ER to the golgi,

where the two proteases are located. In the golgi, S1P cleaves SREBP between its two membrane-spanning segments, therefore dividing the SREBP molecule in half. The NH_2-terminal bHLH-Zip domain is freed from the membrane via a second cleavage mediated by S2P. The NH_2-terminal domain (nuclear SREBP or nSREBP) translocates to the nucleus, where it activates transcription of target genes (e.g. HMGCR) by binding to their SREs in the promoter/enhancer regions (Figure 1.3). When the cholesterol content of cells rises, the SCAP/SREBP complex is retained in the ER so the SREBP precursor does not reach the golgi to be cleaved (Figure1.3). As a result, the transcription of target genes reduces (Brown and Goldstein, 1997; Goldstein *et al*., 2002).

An interesting question is whether cholesterol (end product) inhibits processing of all SREBPs. Studies in cultured human embryonic kidney (HEK-293) cells show that SREBPs are differentially regulated in that cholesterol blocks the processing of SREBP-2 but not SREBP-1 (Hannah *et al*., 2001). Inhibition of SREBP-1 cleavage requires an unsaturated fatty acid, such as oleate, in addition to sterols (Hannah *et al*., 2001). It was suggested that in the presence of sterols but absence of fatty acids, SCAP may be able to escort SREBP-1 proteins (but not SREBP-2) to the golgi, but further studies are required to confirm the mechanism of this differential regulation.

1.5.2. Absorption of Dietary Cholesterol

While cholesterol can be synthesised in the body from acetyl CoA, it is a process that requires considerable energy input. Thus, dietary cholesterol, obtained from foods derived from animal sources that are rich in fat/lipid content (such as eggs, cheese, butter and meat) is absorbed for use by the body, reducing the need for *de novo* cholesterol biosynthesis. The daily intake of cholesterol in humans on a typical Western-style diet is between 300-500 mg (Wang, 2007).

The major sites of dietary cholesterol absorption are the duodenum and proximal jejunum. Most dietary cholesterol is in the form of free sterol, though about 10-15% is esterified as cholesteryl ester (CE; cholesterol esterified to long-chain fatty acids).

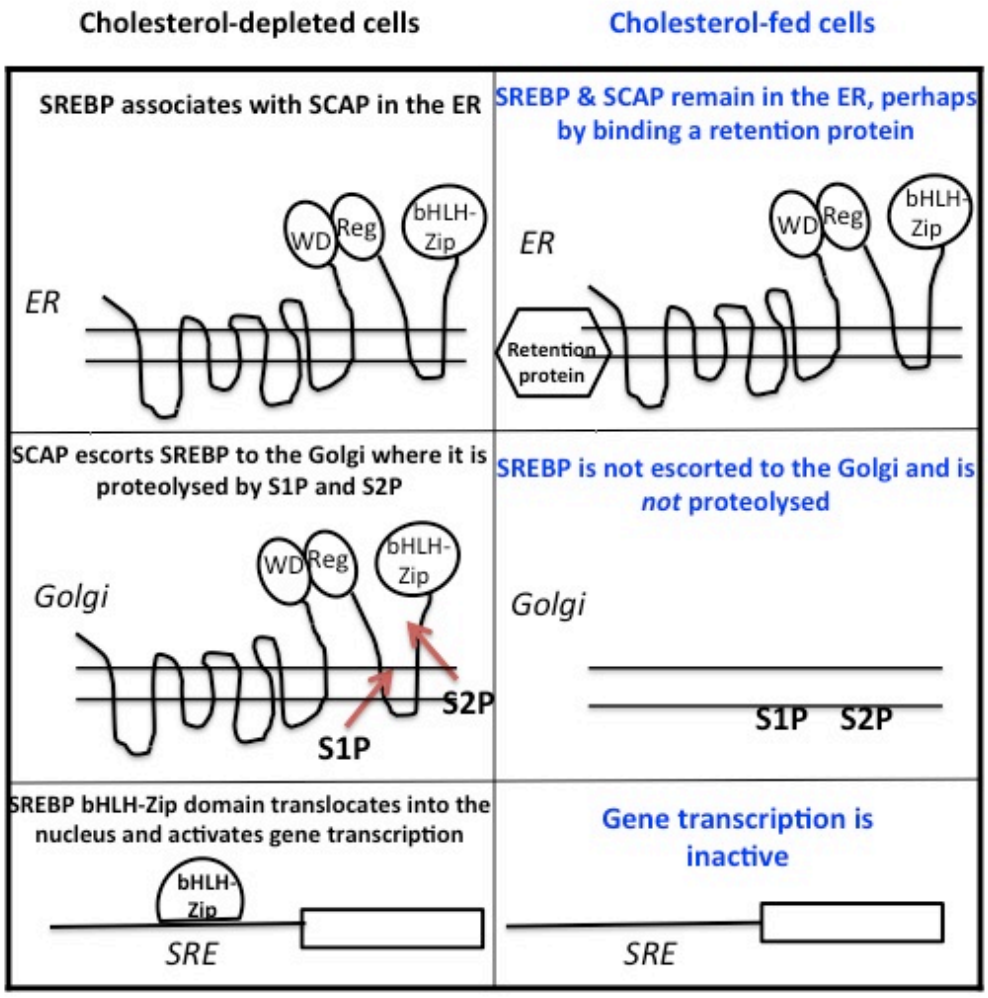


Figure 1.3: SREBP processing and activation

The sterol regulatory element-binding protein (SREBP) precursor is inserted into the endoplasmic reticulum (ER) membrane. The SREBP regulatory domain (Reg) interacts with the SREBP cleavage-activating protein (SCAP), most likely through SCAP's WD repeats. When cholesterol levels are low, SCAP escorts SREBP to the Golgi where the bHLH-Zip domain is released by sequential cleavage, first by S1P, then by S2P. The mature SREBP translocates into the nucleus and activates gene transcription. In cholesterol replete cells, the SREBP precursor and SCAP remain in the ER and the SREBP precursor is not proteolysed to release its bHLH-Zip transcription factor (see text for details). SRE, sterol regulatory element. Figure adapted from Liscum, 2002.

Only non-esterified cholesterol can be absorbed, therefore CEs have to be hydrolysed to release free cholesterol, which can then be absorbed. Cholesterol is almost insoluble in an aqueous environment (Swell *et al*., 1958; Holt *et al*., 1986) so is partitioned into bile salt micelles to be absorbed. Cholesterol usually enters bile salt micelles with triglycerides (TGs), monoacylglycerides, fatty acids, and phospholipids, to form mixed micelles, which are then transported to the brush border of the enterocyte (Westergaard and Dietschy, 1976; Hernell *et al*., 1990; Yao *et al*., 2002).

Cholesterol absorption used to be considered a simple, passive diffusion process. However, it is now recognised that it is transporter-facilitated and cholesterol is selectively taken up by enterocytes with relatively high efficiency compared to structurally similar plant sterols (Monreau *et al*., 2002). There are inter-individual (human) and inter-strain (mouse) variations in the efficiency of cholesterol absorption (Yang *et al*., 1995; Yao *et al*., 2002), and multiple genes participate in cholesterol absorption (Altmann *et al*., 2004; Berge *et al*., 2000; Lee *et al*., 2001; Lu *et al*., 2001).

Genetically modified animal models have provided insights into the transporter-facilitated mechanism of cholesterol absorption. Niemann-Pick C1 like 1 (NPC1L1) is a glycosylated protein localised at the brush border membrane of the enterocyte that acts as a cholesterol uptake transporter (Iyer *et al*., 2005). NPC1L1 has about 50% amino acid homology to NPC1, which is mutated in the cholesterol storage disease, Niemann-Pick disease (Carstea *et al*., 1997). In rodents, NPC1L1 is present only in the small intestine. However, in humans, in addition to the small intestine, NPC1L1 is also located in other tissues such as liver and stomach. Deletion of *Npc1l1* in mice results in reduced intestinal cholesterol absorption (Altmann *et al*., 2004). A SRE in the promoter and a sterol-sensing domain of NPC1L1 appear to regulate cholesterol absorption in response to cholesterol intake; *Npc1l1* expression is suppressed in mice that are fed a cholesterol-rich diet and increased in the cholesterol-depleted porcine intestine (Huff *et al*., 2006).

The scavenger receptor class B type 1 (SR-B1) may also play a role in the control of cholesterol absorption. SR-B1 is localised at both the apical and basolateral membranes of enterocytes (Cai *et al*., 2004). The intestine-specific overexpression of SR-B1 in mice leads to an increase in cholesterol as well as TG absorption in short-

term experiments (Bietrix *et al*., 2006). However, targeted deletion of *Sr-b1* in mice has little effect on cholesterol absorption *in vivo* (Altmann *et al*., 2002; Mardones *et al*., 2001; Wang and Carey, 2002) suggesting that SR-B1 is not essential for intestinal cholesterol absorption.

Once cholesterol is transported into intestinal enterocytes, it can either be pumped back into the lumen via the cholesterol efflux transporters ABCG5/G8 (Berge *et al*., 2000; Lee *et al*., 2001; Lu *et al*., 2001), or it can be esterified for transport within chylomicrons (see below and 1.5.3).

It is worth pointing out that although this section's focus is on absorption of dietary cholesterol, this process of lipid absorption does not occur in isolation in the intestine. Dietary TGs are emulsified by bile acids and hydrolysed by the enzyme lipase, resulting in a mixture of fatty acids and monoglycerides. These then pass from the intestinal lumen into enterocytes, where they are repackaged to form TGs, which further combine with phospholipids, cholesterol ester and apolipoprotein B-48 (see 1.5.3) to form chylomicrons (Carey *et al*., 1983). These chylomicrons pass into lacteals (lymphatic capillaries, found in the villi of the intestine) and are carried to the thoracic duct, where they enter the general circulation. In this way, nearly all dietary lipid is transported in chylomicrons from the gut to the blood through the lymphatic system (Dixon, 2010).

1.5.3. Cholesterol Transport

The cholesterol (with fats) absorbed from the diet is transported in the blood in the form of lipoproteins, with different lipoproteins involved at different stages of cholesterol delivery.

Lipoproteins consist of a hydrophobic cholesteryl ester and TG core surrounded by an outer polar layer of cholesterol, phospholipids and specific apolipoproteins. Apolipoproteins (e.g. apoA, apoB, apoC, apoD or apoE), as well as aiding lipid transport, also facilitate recognition by enzymes, allowing removal of lipids from the particle (Kingsbury and Bondy, 2007).

There are five classes of lipoproteins based on size and density; chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). The composition, density and source of each class of lipoprotein is summarised in Table 1.1.

As mentioned previously, dietary cholesterol is absorbed by intestinal enterocytes, which package it into the TG-rich chylomicrons (Chappell and Medh, 1998; Ginsberg, 1998). Chylomicrons are the largest of the lipoprotein particles and predominantly transport TGs to adipose tissue, but also deliver dietary cholesterol to the liver (Smith *et al*., 1978). The TGs in the chylomicrons are hydrolysed by lipoprotein lipase (LPL), found on the surface of endothelial cells, leaving chylomicron remnants containing mostly cholesterol. The liver rapidly clears these cholesterol remnants (Chappell and Medh, 1998).

The liver repackages cholesterol derived from chylomicron remnants, as well as endogenously synthesised cholesterol with TGs, into VLDL particles for transport to tissues. Like chylomicrons, VLDL is a substrate for LPL. As TGs and cholesterol are delivered to tissues, VLDL particles become less dense to form IDL particles, which are either internalised by the liver or converted to cholesterol-rich LDL (Chappell and Medh, 1998; Ginsberg, 1998).

LDL transports approximately two-thirds of the cholesterol in human plasma. Cells can internalise LDL through cell surface LDL receptors (LDLR). Receptor-mediated endocytosis of LDL and the subsequent liberation of its packaged cholesterol releases cholesterol for use in steroidogenesis or in cell membrane renewal (Brown and Goldstein, 1986). Clearance of circulating LDL requires the hepatic LDLR.

HDL particles, the smallest and densest of the lipoproteins, are produced as protein-rich particles in the liver and small intestine (Millar *et al.*, 1995; Asztalos and Schaefer, 2003) and return excess cholesterol from tissues to the liver. HDL collects cholesterol from cells by interaction with the ATP-binding cassette transporter A1 (ABCA1). Lecithin-cholesterol acyltransferase (LCAT) converts free cholesterol into cholesteryl ester, which is then taken up into the core of HDL particles, thereby

Table 1.1: The source and composition of lipoproteins

	Chylomicrons	VLDL	IDL	LDL	HDL
Density (g/ml)	<0.95	<0.95-1.006	1.006-1.019	1.019-1.063	1.063-1.210
Diameter (nm)	75-1200	30-80	25-35	18-25	5-12
Components (% dry weight) protein	1-2 %	10%	18%	25%	33%
Triglyceride (%)	83	50	31	10	8
Cholesterol and CEs (%)	8	22	29	46	30
Phospholipids (%)	7	18	22	22	29
Apolipoprotein composition	A-I, A-II, B-48, C-I, C-II, C-III	B-100, C-I, C-II, C-III, E	B-100, C-I, C-II, C-III, E	B-100	A-I, A-II, C-I,II,III, D, E
Source	Intestine	Liver	Catabolism of VLDL	Catabolism of IDL	Liver, Intestine

There are five different classes of lipoproteins based on their size and density; chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). This table summarises the composition and source of the different lipoproteins. Source of information: Arias *et al*., 2009.

increasing the size of HDL particles as they circulate through the bloodstream. In humans, esterified cholesterol can be transferred from HDL to VLDL and LDL particles, via cholesteryl ester transfer protein (CETP), for delivery to steroidogenic tissues such as the adrenal glands and gonads, or returned to the liver for excretion of cholesterol in bile (Assmann and Gotto, 2004). The transfer of cholesterol from extra-hepatic tissues to the liver is termed reverse cholesterol transport.

1.5.4. Cholesterol Efflux

Sterol removal is vital for the maintenance of cellular cholesterol balance. Peripheral cells do not break down cholesterol so their only mechanism for removal is via cholesterol efflux and reverse cholesterol transport (RCT) (Glomset and Wright, 1964; Glomset, 1968).

The specific process of cholesterol efflux from cholesterol-enriched macrophages in atherosclerotic lesions has been termed macrophage reverse cholesterol transport (Cuchel and Rader, 2006). The adenosine triphosphate (ATP) binding cassette transporters A1 (ABCA1) and G1 (ABCG1) are the key molecules involved in cholesterol efflux from macrophage foam cells. ABCA1 promotes cholesterol as well as phospholipid efflux onto lipid-poor apolipoproteins, apoA-1 or apoE (Rust *et al*., 1999; Bodzioch *et al*., 1999; Brooks-Wilson *et al*., 1999; Wang *et al*., 2000). Some studies show that, in addition to its role in cholesterol efflux from arterial wall cells, ABCA1 is also responsible for the initiation of HDL particle formation in the liver and small intestine (Timmins *et al*., 2005; Brunham *et al*., 2006). ABCG1 promotes efflux of free cholesterol onto HDL particles (Wang *et al*., 2004; Kennedy *et al*., 2005). Within HDL particles, the enzyme LCAT esterifies some of the free cholesterol. In humans, CETP transfers esterified cholesterol to TG-rich lipoproteins, which are subsequently removed from the circulation when LDL receptors or heparin sulphate proteoglycans mediate the hepatic uptake of remnant lipoproteins (MacArthur *et al*., 2007). In rodents, and possibly humans, free and esterified cholesterol in HDL particles is directly taken up into the liver by SR-B1, in a process of selective uptake (Acton *et al*., 1996; Rinninger *et al*., 1994). The above-mentioned process of cholesterol efflux not only operates to remove cholesterol from

macrophage-derived foam cells, but also from other peripheral cells.

The relative contributions of the different cholesterol efflux mechanisms have been determined in mouse macrophages; aqueous diffusion, 35%; ABCA1, 35%; ABCG1, 21%; and SR-B1, 9% (Adorni *et al*., 2007). On the other hand, in human macrophages, the SR-B1 pathway is relatively more important, and ABCG1 does not contribute to efflux (Larrede *et al*., 2009).

The complicated process of RCT allows the removal of cholesterol from peripheral cells for delivery to the liver for faecal excretion.

1.5.5. Cholesterol Excretion

Cholesterol is excreted via a number of pathways: minimal amounts are excreted through skin and intestinal cell shedding (Bhattacharyya *et al*., 1972; Ferezou *et al*., 1981; Bhattacharyya *et al*., 1983), with the majority excreted via biliary (or hepatobiliary) cholesterol secretion (Bloch *et al*., 1943; Dikkers and Tietge, 2010), which is considered to be the final step in the RCT pathway. Once excess cholesterol from the periphery is brought into the liver, the liver secretes cholesterol into bile either directly as free cholesterol or after conversion into bile acids, therefore resulting in irreversible elimination of cholesterol from the body via the faeces (Angelin *et al*., 2002; Lewis, 2006).

Direct secretion of cholesterol into bile takes place in a process involving the ATP-binding cassette transporters, ABCG5 and ABCG8 (Berge *et al*., 2000; Graf *et al*., 2003). This heterodimer transporter pair is expressed in the liver and intestine (Klett *et al*., 2004b). In humans, mutations in both have been identified, leading to sitosterolaemia, an autosomal recessive disorder characterised by the accumulation of plant sterols in blood and tissues as well as decreased biliary sterol secretion (Hazard and Patel, 2007; Scriver *et al*., 1995). In the intestine, ABCG5 and ABCG8 efflux/secrete absorbed plant sterols back into the intestinal lumen (see 1.5.2), whereas in the liver, ABCG5/G8 promote sterol efflux into the bile canaliculus, for secretion in bile (Yu *et al*., 2002a; Yu *et al*., 2002b) (Figure 1.4). Supporting this,

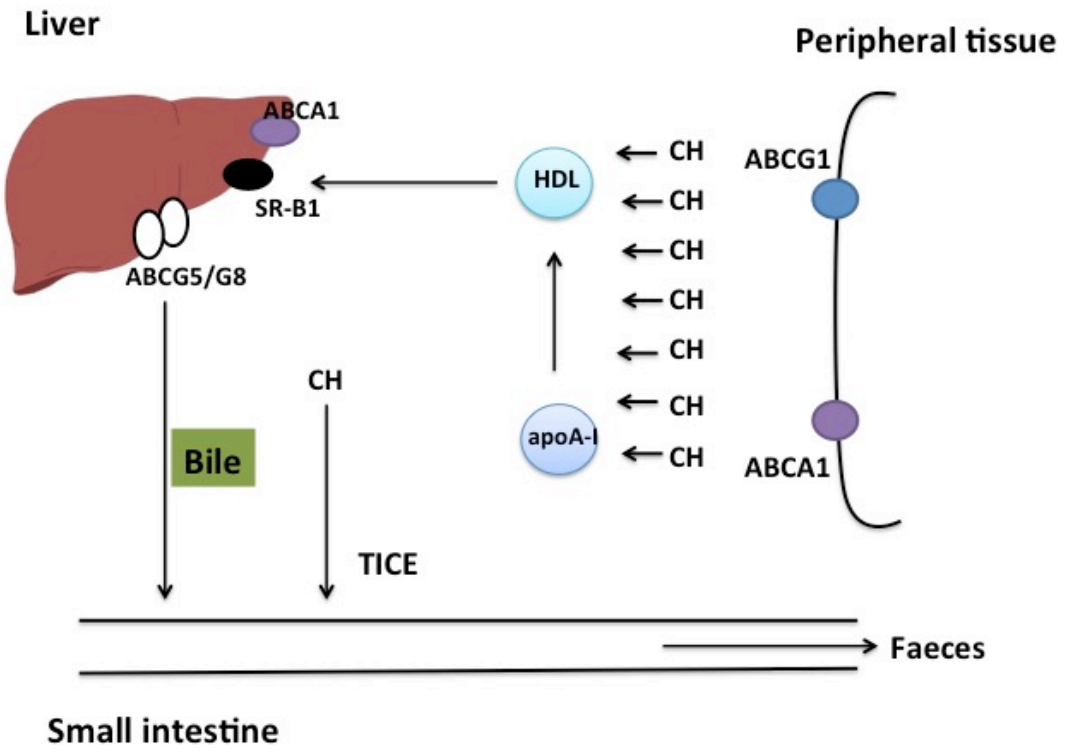


Figure 1.4: A schematic overview of the major cholesterol excretion pathways.

Refer to above text for detailed description on cholesterol efflux and excretion. ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; ABCG5, ATP-binding cassette transporter G5; ABCG8, ATP-binding cassette transporter G8; apoA-I, apolipoprotein A-I; CH, cholesterol; HDL, high density lipoprotein; Sr-B1, scavenger receptor class B type 1; TICE, transintestinal cholesterol efflux. Figure adapted from Vrins, 2010.

biliary cholesterol secretion is reduced by approximately 75% in ABCG5 and/or ABCG8 knockout mice (Yu *et al*., 2002b; Klett *et al*, 2004a; Plösch *et al.*, 2004). On the other hand, biliary cholesterol secretion is significantly increased in mice with transgenic overexpression of ABCG5/G8 (Yu *et al*., 2002a; Yu *et al*., 2005).

The liver can also eliminate cholesterol from the body via hepatic conversion of cholesterol into bile acids (Russell and Setchell, 1992; Chiang and Vlahcevic, 1996; Chiang, 2002). Bile acids are amphipathic molecules, containing hydrophobic and hydrophilic properties, and a negative charge at one end (Hofmann *et al*., 2008). Therefore, unlike water-insoluble cholesterol, bile acids are water-soluble and readily excreted. In humans, the major primary bile acids include cholic acid (CA) and chenodeoxycholic acid (CDCA). In mice, CA and β-muricholic acid (MCA) are the main primary bile acids (Ziboh *et al*., 1961).

Bile acid synthesis takes place in hepatocytes via two pathways, the classic (or neutral) pathway and the alternative (or acidic) pathway (Russell, 2003). In humans, the classic pathway produces CA and CDCA in equal amounts. Cholesterol 7α-hydroxylase, encoded by CYP7A1, catalyses the first, rate-limiting, step of the classic pathway (Myant and Mitropoulos, 1977). The alternative pathway mainly produces CDCA (Javitt *et al*., 1989; Javitt, 1994), and in this pathway, sterol 27-hydroxylase, encoded by CYP27A1, catalyses the first reaction (Pikuleva *et al*., 1998). Studies in genetically modified mice and in rats reveal that the percentage of total daily bile acid production via the alternate pathway is significantly greater in these animal models than that found in humans (Vlahcevic *et al*., 1997; Rosen *et al*., 1998; Schwarz *et al*., 1998; Duane and Javitt, 1999; Li-Hawkins *et al*., 2000; Repa *et al*., 2000a).

Although the liver is the major organ involved in cholesterol metabolism and excretion, evidence supports a role for the small intestine in active cholesterol excretion, in a process called transintestinal cholesterol efflux (TICE) (Temel and Brown, 2010; van der Velde *et al*., 2010; Vrins, 2010; Brufau *et al*., 2011). Interestingly, despite their significantly reduced biliary cholesterol secretion, ABCG5/G8 double knockout mice do not show the expected low faecal neutral sterol output (Yu *et al*., 2002b), suggesting that biliary cholesterol secretion might not be

the only cholesterol excretion route. Faecal sterols of non-dietary origin have been observed in rats with long-term bile diversion (Bandsma *et al*., 2000) as well as in patients with biliary obstruction (Cheng and Stanley, 1959). Performing intestine perfusions in mice that were injected with radiolabelled cholesterol confirmed the presence of the TICE pathway as the direct secretion of cholesterol from blood through the intestinal wall into the intestinal lumen was established (van der Velde *et al.*, 2007; van der Veen *et al*., 2009). The complete length of the small intestine can secrete considerable amounts of cholesterol, but this secretion is most significant at the proximal end. It has also been revealed that, in mice, the TICE pathway accounts for nearly 33% of total neutral sterol loss (van der Veen *et al*., 2009). In humans, although there are studies showing the role of the gut in cholesterol excretion (Cheng and Stanley, 1959; Simmonds *et al*., 1967), the relative contribution of this non-biliary pathway to total sterol output remains to be established (Temel and Brown, 2012).

The liver X receptors (LXRα and LXRβ) regulate cholesterol homeostasis, specifically with respect to the RCT pathway. The liver X receptors are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors, and their activation promotes cholesterol efflux from peripheral cells as well as cholesterol excretion from liver, and inhibition of intestinal cholesterol absorption (Calkin and Tontonoz, 2010). LXR activation increases transcription of ABCA1 and ABCG1, increasing cholesterol efflux, and hepatic ABCG5, ABCG8, CYP7A1 aiding cholesterol excretion (Lehmann *et al*., 1997; Peet *et al*., 1998; Repa *et al*., 2002; Yu *et al*., 2003; Calkin and Tontonoz, 2010). LXR activation also inhibits cholesterol absorption because activation of ABCG5/8 in the small intestine limits intestinal cholesterol absorption. Animal studies have also shown LXRα to be predominant in the regulation of RCT in mice (Peet *et al*., 1998; Alberti *et al*., 2001; Zhang *et al*., 2012).

1.6. Cholesterol Homeostasis and Cardiovascular Disease

When intracellular cholesterol levels exceed cellular demand and removal processes, the excessive build-up of cholesterol can disrupt cell membranes and promote apoptosis (Feng *et al*., 2003). Moreover, cholesterol accumulation in cells of the artery wall can lead to atherosclerotic cardiovascular disease, which is a leading cause of morbidity and mortality worldwide (Yuan *et al*., 2006; Cullen *et al*., 2005).

The term 'atherosclerosis' is derived from the Greek words "athero" referring to paste or gruel, and "sclerosis" referring to hardness. Atherosclerosis results from the accumulation of fatty material along the inner artery wall lining, and is the primary cause of heart disease. The fatty deposits may increase in size, developing into plaque or lesions, which are mainly composed of cholesterol-enriched macrophages (foam cells) (Cullen *et al*., 2005; Maxfield and Tabas, 2005; Pennings *et al*., 2006). This eventually leads to hardening and narrowing of the blood vessel, and when unstable plaques rupture, it can result in a life-threatening myocardial infarction or stroke (Cullen *et al*., 2005; Maxfield and Tabas, 2005).

Patients with familial hypercholesterolaemia, a rare inherited disorder, accumulate extremely high levels of blood cholesterol from early childhood and develop coronary heart disease in youth, despite absence of other risk factors such as high blood pressure or smoking (Yuan *et al*., 2006). Elevated levels of plasma LDL cholesterol and decreased levels of HDL cholesterol are associated with the development (and progression) of atherosclerosis (Libby, 2002; Brewer and Santamarina-Fojo, 2003; Hersberger and von Eckardstein, 2003; Cullen *et al*., 2005; Pennings *et al*., 2006). The Framingham Heart Study is an ongoing study on the residents of the town of Framingham in Massachusetts, USA, which started with approximately 5200 adult subjects (Mahmood *et al*., 2013). This study was one of the first studies developed to identify and assess risk factors for cardiovascular disease. Amongst other risk factors, this study showed that as total cholesterol levels rise above 200 mg/dl, heart disease risk increases accordingly. Another major finding of this study is that high levels of HDL cholesterol reduce risk of heart disease (Mahmood *et al*., 2013). In the Seven Countries Study, the dietary intake and heart disease rates of seven countries (USA,

Finland, Netherlands, Italy, Greece, Former Yugoslavia, Japan) and 16 population subgroups were examined, showing a positive correlation between the intake of dietary cholesterol and blood cholesterol levels (and, ultimately, heart disease rates) (Kromhout, 1999). Plasma cholesterol levels and its distribution among lipoproteins are predictive tools to assess risk of a cardiac event, and are routinely used in the clinics. Cholesterol homeostasis (or its deregulation) plays a crucial role in the initiation of atherosclerosis (Yuan *et al*., 2012).

Reduction of hepatic cholesterol synthesis is an attractive therapeutic approach. Statins are competitive inhibitors of HMGCR, the rate-limiting enzyme in the cholesterol biosynthetic pathway, and are used in therapies against hypercholesterolaemia and atherosclerosis (Shepherd *et al*., 2002; De Bacquer *et al*., 2003; Baigent *et al*., 2005). However, the use of statins can have severe side-effects, the most frequent being myopathy, ranging from myalgia to fatal rhabdomyolysis (Hamilton-Craig, 2001; Rozman and Monostory, 2010).

Preventing the absorption of dietary cholesterol can also help control plasma cholesterol levels in hypercholesterolaemic conditions. Ezetimibe is a drug that does exactly this, in both humans and mice, through NPC1L1 inhibition (Davis *et al*., 2002). Although cholesterol absorption inhibition by ezetimibe has shown to stimulate a compensatory increase in endogenous cholesterol biosynthesis (Davis *et al*., 2002; Davis and Veltri, 2007), ezetimibe monotherapy lowers plasma LDL cholesterol concentrations by approximately 15-20% (Sudhop *et al*., 2002; Dujovne *et al*., 2002). Trials have also shown additional cholesterol-lowering benefit by combining ezetimibe with statin therapy (Mikhailidis *et al*., 2011). Additionally, ezetimibe has also shown to stimulate RCT from macrophages in mice (Sehayek and Hazen, 2008; Briand *et al*., 2009).

As one of the major killers in the Western and increasingly the developing world, it is important to understand the (patho)physiology of atherosclerosis for continued identification of therapeutic targets. Oxysterols have been identified as modulators of cholesterol metabolism and also been linked with atherosclerosis.

1.7. Oxysterols in Cholesterol Metabolism and Atherosclerosis

Oxysterols are oxygenated cholesterol derivatives that are formed either by enzymatic or non-enzymatic reactions. In mammals, oxysterols are always present at very low concentrations compared to cholesterol (Brown and Jessup, 1999). Oxysterols fall into two main categories, 1) those that are oxygenated on the sterol ring, including 7α/β-hydroperoxycholesterol, 7-ketocholesterol (7-KC) and 7α/β-hydroxycholesterol (7α-HC; 7β-HC), and 2) those that are oxygenated on the side chain, e.g. 24S-hydroxycholesterol, 25-hydroxycholesterol and 27-hydroxycholesterol (Schroepfer Jr., 2000).

As natural ligands for LXRs, oxysterols have been shown to stimulate downstream cholesterol efflux and excretion pathways, as well as limit lipoprotein uptake (Janowski *et al*., 1996; Zelcer *et al*., 2009). Oxysterols also directly inhibit processing of SREBPs, thereby inhibiting the cholesterol biosynthesis pathway (Schmidt *et al*., 2006; Radhakrishnan *et al*., 2007).

Despite making up less than 0.1% of the total cholesterol in the body, oxysterols are major components of oxidised LDL (nearly 60%) and, therefore, are highly abundant in the plasma and vessel wall (Dzeletovic *et al*., 1995; Brown and Jessup, 1999). Both 7-KC and 7β-HC are prominent in atherosclerotic plaques (Hughes *et al*., 1994; Brown and Jessup, 1999; Jessup and Brown, 2005; Larsson *et al*., 2006). Unlike normal physiological levels, the oxysterol:cholesterol ratio in atherosclerotic plaques is much higher (Bjorkhem *et al*., 1994; Zieden *et al*., 1999). There is also evidence that 7β-HC is possibly more toxic than 7-KC (Steffen *et al*., 2006).

1.8. A Role for 11β-HSD1 in 7-Oxysterol Metabolism

As well as catalysing the intracellular conversion of inactive GCs to active GCs, the enzyme 11β-HSD1 also interconverts 7-oxysterols in some animal models, catalysing the reduction of 7-KC to 7β-HC (Schweizer *et al*., 2004; Hult *et al*., 2004; see 1.3). As these 7-oxysterol substrates competitively inhibit GC metabolism, they could be

important regulators of intracellular GC levels in tissues where they accumulate to substantial levels such as liver, adipose tissue and brain (Wamil *et al*., 2008; Chapman *et al*., 2013).

Interestingly, 7-KC inhibits cleavage of SREBP-2, whereas 7β-HC does not (Brown *et al*., 2002). In liver microsomes of mice transgenically overexpressing hepatic 11β-HSD1 (LOE mice), there are significantly reduced levels of 7-KC compared to those found in control mice (Dr. T. Mitic, unpublished data, shown in Chapter 3 of this thesis). Moreover, preliminary data point out to LOE liver microsomes having increased levels of cleaved SREBP-2 protein compared to control mice, suggesting increased *de novo* cholesterol synthesis (Dr. T. Mitic and K. Manwani, unpublished data; see 3.1). These findings, along with the evidence for altered cholesterol handling in HF-fed LOE mice (Paterson *et al*., 2004), form the basis for the following hypothesis.

1.9. Hypothesis and Aims

Hypothesis: Hepatic 11β-HSD1 converts 7-KC to 7β-HC, removing an endogenous brake on cholesterol synthesis. Thus, hepatic 11β-HSD1 promotes *de novo* cholesterol biosynthesis. This hypothesis predicts that hepatic 11β-HSD1 plays a role in cholesterol homeostasis: elevated 11β-HSD1 levels in liver are predicted to increase cholesterol synthesis whereas liver-specific 11β-HSD1 deficiency is predicted to decrease cholesterol biosynthesis.

In order to test this hypothesis, the following aims were set:

1. To examine the *in vivo* effects of liver 11β-HSD1 overexpression on cholesterol biosynthetic and excretion pathways.

2. To study the *in vivo* effects of 11β-HSD1 on cholesterol synthesis and clearance pathways when substrate for lipid/cholesterol is increased.

3. To examine the *in vivo* effects of liver-specific 11β-HSD1 deficiency on cholesterol homeostasis.

Chapter 2. Materials and Methods

2.1. Materials

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich, U.K.

2.1.1. General Chemicals

Table 2. 1 General chemicals

Anti-SREBP2 Antibody, rabbit polyclonal (ab112046)	Abcam
Agilent 600 RNA Nano Kit	Agilent Technologies
^{3}H-corticosterone	Amersham Pharmacia Biotech
D_c Protein Assay Blotting-grade Blocker Non-fat Dry Milk	Bio-Rad Laboratories Ltd.
GelRed Nucleic Acid Gel Stain	Biotium, Inc.
Rainbow™ Full-Range Molecular Weight Marker	GE Healthcare
SuperScript® III Reverse Transcriptase 1kB DNA Ladder TRIzol® Reagent Alexa Fluor® 700 Goat Anti-rabbit IgG Alexa Fluor® 800 Donkey Anti-sheep IgG NuPAGE® LDS Sample Buffer NuPAGE® Sample Reducing Agent NuPAGE® Transfer Buffer NuPAGE® MES SDS Running Buffer NuPAGE® Antioxidant Novex® 4-12% Bis-Tris Gels	Invitrogen
IRDye® Goat Anti-rabbit 800CW IgG IRDye® Goat Anti-mouse 800CW IgG IRDye® Goat Anti-mouse 680 IgG	LI-COR Biosciences
SeaKem™ LE Agarose	Lonza
Anti-β-tubulin Antibody, mouse monoclonal	Millipore
100bp DNA Ladder	New England Biolabs
dNTPs RNaseIN® Plus Random Primers GoTaq® DNA Polymerase 1kb DNA Ladder	Promega
RNeasy® Mini Kit DNeasy® Blood&Tissue Kit	Qiagen
LightCycler® 480 Probes Master Universal ProbeLibrary Probes cOmplete Protease Inhibitor Cocktail	Roche Applied Science
Cryo-M-Bed Embedding Medium	TAAB Laboratories Equipment
PermaFluor™ Aqueous Mounting Medium	Thermo Scientific

2.1.2. Miscellaneous Equipment

Table 2. 2 Miscellaneous equipment

Agilent 2100 Bioanalyzer	Agilent Technologies
Ultracentrifuge J2-MC	Beckman Coulter
Power Pack 200 Agarose Gel System	Bio-Rad
pH Meter BASIC AA-160 Balance	Denver Instrument
Eppendorf 5415 R Centrifuge Eppendorf 5810 R Centrifuge	Eppendorf
Surgical instruments	Fine Science Tools Inc.
Rotamixer Vortex	Hook&Tucker Instruments
T10 Ultra-Turrax® Basic Homogeniser	IKA
XCell SureLock™ Mini-Cell Electrophoresis System	Invitrogen
3510 pH Meter	Jenway
Leica CM1900 Cryostat	Leica
Odyssey® Infrared Imaging System	LI-COR Biosciences
Rainin EDP3-Plus Electronic Multichannel Pipettes	Mettler Toledo
Milli-Q® Integral Water Purification System Synringe 0.2μm filters	Millipore
OPTImax Tunable Microplate Reader	Molecular Devices
NanoDrop ND-1000 Spectrophotometer	NanoDrop Technologies
LightCycler® 480 384-well qPCR Plates and Plastic Covers	Roche Applied Science
0.2ml Strip-tubes with lids	StarLab
Stuart® Heat-stir CB 162 Platform Stuart® Gyro-rocker SSL3 Platform Stuart® SB2 Rotator	Stuart Equipment
Disposable scalpels	Swann-Morton
Ori-Block DB-3 Heating Block HB-1D Oven Hybridiser Thermal Cycler TC-412 Thermal Cycler TC-512	Techne
Savant DNA110 SpeedVac® Concentrator HistoStar Embedding Workstation Nunclon™ Surface 96-well Plates Tissue Flotation Bath Sterilin® 90mm Petri Dishes	Thermo Scientific
UviPro System	UviTec
Vacuum Gas Pump SuperFrost® Plus Microscopic Slides Cover Glass Rectangular 22x50mm Thickness No.1	VWR International Ltd.
Whatman® paper No.1	Whatman
Zeiss Axioscop microscope	Zeiss

2.1.3. Software

Table 2. 3 Analysis software

Means of acquisition	Software
Agilent 2100 Bioanalyzer	2100 Expert Software (Agilent Technologies)
LightCycler® 480	LightCycler® 480 release 1.5 O SP3 (Roche)
NanoDrop ND-1000 spectrometer	ND-1000 v3.3.0 (NanoDrop Technologies)
Odyssey® Infrared Imager	Odyssey v3.0 (LI-COR Biosciences)
OPTImax Tunable Microplate Reader	SoftMax Pro 4.8 (Molecular Devices)
Statistical analysis and Image processing	GraphPad Prism 5 (GraphPad)
UviPro System	UviPro 12.4 (UviTec)
Zeiss Axioscop microscope	KSM 300 3.0 (Zeiss)

2.1.4. Solutions and Buffers

All solutions were prepared with Milli-Q® water unless stated otherwise. All buffers were stored at 4°C.

Blocking buffer: 5% w/v Blotting-grade non-fat dry milk in TBST

'C' buffer: Dissolve 63g glycerol, 8.77g NaCl, 186mg ethylenediaminetetraacetic acid (EDTA) and 3.03g Tris in 500ml water; pH 7.7

Coomassie stain: 45% v/v methanol, 10% v/v acetic acid, 0.25% w/v Coomassie blue dye

Coomassie destain solution: 45% v/v methanol, 10% v/v acetic acid

DEPC-water: 0.1ml of diethylpyrocarbonate (DEPC) per 100ml, mix well, leave for 1-25h, autoclave

DNA ladder: 25% v/v 1kb DNA ladder (Promega), 25% v/v TE buffer, 50% v/v Orange G loading dye

DNA/RNA loading buffer: 0.3% w/v Orange G, 40% v/v glycerol

Homogenisation buffer: Dissolve 100g glycerol, 300mg Tris and 186mg EDTA in 500ml water; pH 7.5

Lysis buffer: 25mM HEPES, 68.5mM NaCl, 0.5mM $MgCl_2$, 0.5mM $CaCl_2$, 5mM NaF, 1mM EDTA, 5mM sodium pyrophosphate, 1% NP-40, 10% glycerol, 1X protease inhibitor cocktail (PIC; see below)

MOPS (10X): 0.4M 3-(N-morpholino)propanesulfonic acid (MOPS), 0.1M sodium acetate, 10mM EDTA; pH 7.0

NADP: 1.1mg nicotinamide adenine dinucleotide sodium salt in 1684µl 'C' buffer (final concentration of 2mM)

Paraformaldehyde (4%): Bring water/PBS to 80°C, add 4% w/v paraformaldehyde and stir at 80°C until dissolved

PIC (Protease Inhibitor Cocktail, 25X): Dissolve a table of complete protease inhibitor cocktail (Roche Applied Science) in 2ml respective solution

Running buffer (SDS-PAGE): 1X NuPAGE® MES SDS Running buffer (Invitrogen), 0.05% v/v NuPAGE® Antioxidant (Invitrogen)

TBE (10X): 1.1M Tris, 0.9M boric acid, 12.5mM EDTA

TBS (10X): 0.2M Tris, 1.37M NaCl; pH 7.6

TBST: 0.1% v/v Tween-20 in 1X TBS

TE: 10mM Tris pH 8.0, 1mM EDTA

Transfer buffer: 1X NuPAGE® Transfer buffer (Invitrogen), 20% v/v methanol, 0.1% NuPAGE® Antioxidant (Invitrogen)

2.1.5. Animals

All animal experiments were carried out according to the accepted standards of humane animal care under the auspices of the United Kingdom Animals (Scientific Procedures) Act 1986, after prior approval by the University of Edinburgh Animal Ethical Review Committee. All experimental procedures were performed under a UK Home Office Project Licence (Seckl, 60/3962) and personal licence (Manwani, 60/12840).

All mice were bred and maintained in the Biomedical Research Facility, Little France, Edinburgh, U.K., unless stated otherwise. Mice were kept in groups of 1-8 per cage under controlled conditions (12h light/dark cycle; 21°C), with *ad libitum* access to water and standard rodent chow diet (Special Diet Services, U.K.), except in the case of diet experiments (section 2.2.3).

For the experiments conducted during preparation of this thesis the following strains of mice were used:

- C57BL/6 mice (in-house stock)
- "Floxed" *Hsd11b1* mice: *Hsd11b1*$^{flox/flox}$ mice (generated by a commercial supplier, TaconicArtemis; see Appendix for further details; in-house stock)
- Mice transgenically overexpressing 11β-HSD1 in the liver: LOE mice (Paterson *et al*., 2004; in-house)
- 11β-HSD1 deficient mice ("old knockout"): HSD1 KO (Kotelevtsev *et al*., 1997; Morton *et al*., 2004; in-house)
- Newer global 11β-HSD1 knockout mice: Del (generated in-house by crossing *Hsd11b1*$^{flox/flox}$ mice with Hprt-Cre mice; unpublished)
- C57BL/6-Tg(Alb-cre) transgenic mice, stock number: 003574 (Postic *et al*., 1999; ordered from The Jackson Laboratory, U.S.A.)
- Liver-specific 11β-HSD1 knockout mice: LKO (generated as part of this study; see 2.2.1, and Chapter 5)

2.2. Methods

2.2.1. Generation of LKO mice

In order to generate liver-specific 11β-HSD1 knockout (LKO) mice, the Cre-lox system was used. C57BL/6-Tg(Alb-cre) transgenic mice (one male, and one female) were ordered from The Jackson Laboratory, U.S.A. (JAX Mice Database- 003574 B6.Cg-Tg(Alb-cre)21Mgn/J; http://jaxmice.jax.org/strain/003574.html). These Alb-cre mice were made by injecting a transgene containing a 2.34 kb mouse albumin enhancer/promoter, nuclear localisation sequence-modified Cre recombinase, and a 2.1 kb fragment from the human growth hormone, into the pronuclei of B6D2(F2) mice, with subsequent backcrossing to C57BL/6 mice (Postic *et al*., 1999).

Alb-cre mice were bred to *Hsd11b1*$^{flox/flox}$ mice. The *Hsd11b1*$^{flox/flox}$ mice have exon 3 of the *Hsd11b1* gene flanked by *loxP* sites, allowing Cre-driven recombination (Figure 2.1A). The resulting F1 progeny were bred to *Hsd11b1*$^{flox/flox}$ to generate a line of liver-specific 11β-HSD1 knockout mice, LKO (Figure 2.1B). Offspring were genotyped by polymerase chain reaction (PCR) analysis of ear notch or tail DNA (see 2.2.2 for genotyping protocol and reactions); Cre genotyping identified presence (or absence) of the Cre transgene (Figure 2.2A) and "Flox" genotyping was carried out to check the homozygous state of the "floxed" *Hsd11b1* gene (Figure 2.2B).

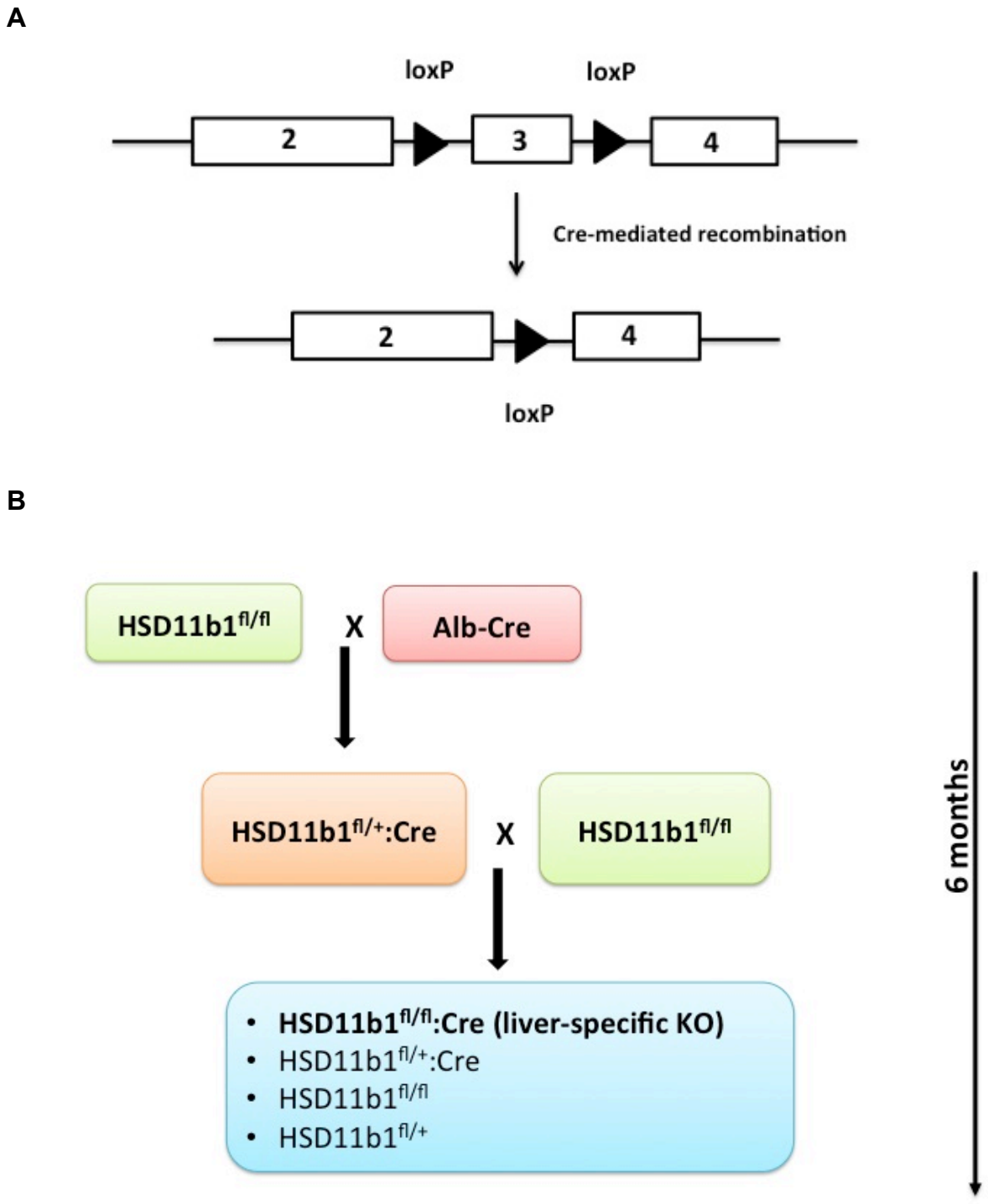


Figure 2.1: A schematic representation of Cre-mediated recombination at the Hsd11b1 allele, and a scheme showing the generation of mice with liver-specific knockout of Hsd11b1 (LKO mice).

(A) Cre-mediated recombination leads to deletion of the "floxed" exon 3 of the *Hsd11b1* allele. **(B)** Two rounds of breeding were required to produce mice with liver-specific knockout of *Hsd11b1* (~3 months/breeding round). All mice were bred and maintained in the Biomedical Research Facility, Little France, Edinburgh, U.K.

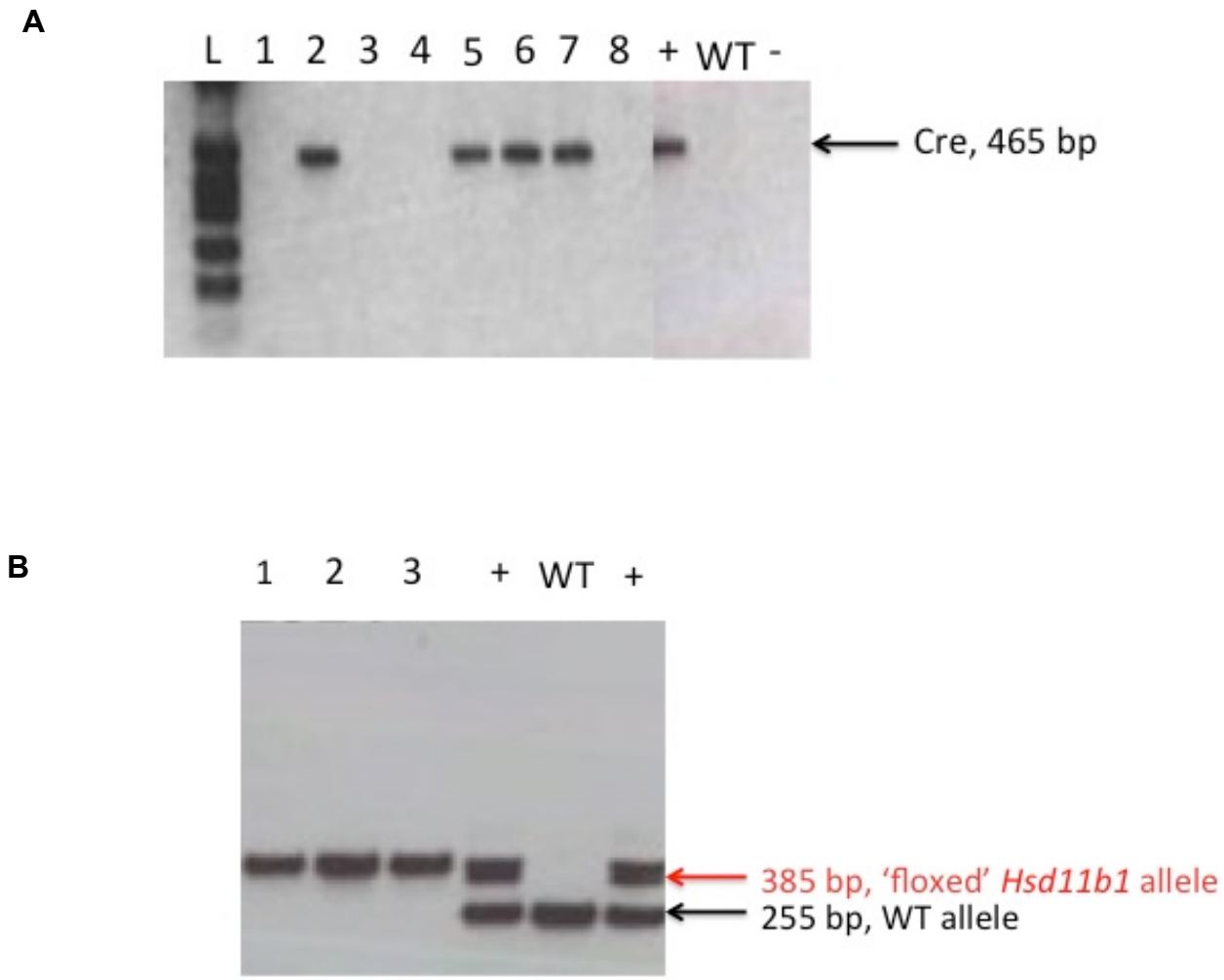


Figure 2.2: Representative gel images showing genotyping of offspring from the cross between Alb-Cre and *Hsd11b1*$^{flox/flox}$ mice.

(A) Genotyping for Cre transgene (465bp) showed that mice 2, 5, 6 and 7 are positive for Cre. '+' indicates a positive control, 'WT' wild-type control (negative for Cre) and '-' indicates H_2O control. 'L' indicates 1kb DNA ladder. **(B)** Genotyping for the 'floxed' allele of *Hsd11b1* (385 bp for floxed *Hsd11b1*, 255 bp for wild-type allele) showed that mice 1, 2 and 3 were homozygous (flox/flox) for the 'floxed' allele. '+' indicates samples from heterozygous mice (flox/+), 'WT': wild-type control. Taken together, data from (A) and (B) show that mouse 2 (*Hsd11b1*$^{flox/flox}$:Cre) is a liver-specific 11β-HSD1 knockout.

2.2.2. Genotyping

Animals were genotyped by PCR using DNA obtained from their frozen tissues.

2.2.2.1. Tissue DNA Extraction using Spin-Column Protocol

DNA was extracted from a frozen piece of tissue (tail or ear clips from weaned animals) using a DNeasy® Tissue&Blood kit (Qiagen, UK). Tissue was incubated with 180µl of AL buffer (Qiagen, supplied with the kit) supplemented with 20µl of proteinase K solution (supplied with the kit) at 56°C, with rotation, until completely lysed (usually ~4 h). Tubes were then vortexed and a further 200µl of AL buffer was added, followed by 200µl of 96% ethanol. Contents were thoroughly mixed and transferred to a spin column placed in a 2ml collection tube. Tubes were centrifuged at 8000g, room temperature for 1 min. The column was then placed into a new 2ml collection tube, and 500µl of AW1 buffer (Qiagen, supplied with the kit) was added, followed by centrifugation as above. The column was again placed into a new 2ml collection tube and 500µl of AW2 buffer (Qiagen, supplied with the kit) was added, followed by centrifugation at 12,000g, room temperature for 3 min. The column was then placed into a 1.5ml tube and 100µl of AE buffer (Qiagen, supplied with the kit) was added. Samples were incubated at room temperature for 1 min, and then centrifuged at 8000g for 1 min to elute the DNA. DNA was processed immediately or stored at -20°C.

2.2.2.2. PCR analysis of Transgene Expression

PCR reactions for each DNA sample were carried out using a Qiagen Taq kit. 1.5µl DNA was added to 2.5µl $MgCl_2$-free buffer (supplied with the kit), 2.25µl $MgCl_2$ (supplied with the kit), 1µl dNTP mix (10mM), 1µl forward primer (10pmol/µl), 1µl reverse primer (10pmol/µl), 0.2µl Taq DNA polymerase (Qiagen, UK), and 15.55µl nuclease-free water. Specific primer sequences, PCR conditions, and expected product lengths are listed in Table 2.4. In each case, the final step comprised a final extension at 72°C for 10 min followed by 4°C hold. PCR products were detected by agarose gel electrophoresis as described in (2.2.9).

Table 2. 4 List of PCR primers and reaction conditions

	Cre	*Flox*
Primers		
Forward (5'-3')	gaccgtacaccaaaatttgcctg	cttgcatgtgtttggtgttgg
Reverse (5'-3')	ttacgtatatcctggcagcgatc	cccacaatgcatttggaaacatt
PCR		
Denature	94°C, 10 minutes	96°C, 10 minutes
No. of cycles	32	35
Step1	94°C, 1 minute	95°C, 30 seconds
Step2	64°C, 1 minute	60°C, 30 seconds
Step3	72°C, 1 minute	72°C, 1 minute
Product		
Size	465bp	WT: 255bp
		Flox: 385bp

2.2.3. Dietary Experiments

Young adult (5-6 week old) male mice (n = 6-8/group) were fed one of the following diets; chow diet (Special Diet Services, U.K.), 58% high fat (D12331; Research Diets Inc., U.S.A.) or western diet (0.21% cholesterol and 41% fat; D12079B; Research Diets Inc., U.S.A.), for 12 weeks. Diet constituents are shown in Tables 2.5 (chow), 2.6 (high fat) and 2.7 (western). Body weight was measured weekly, and, at the end of the experiment, mice were killed by cervical dislocation. Blood was collected in EDTA tubes for plasma collection. The following tissues were harvested at sacrifice, snap frozen on dry ice and weighed. Whole livers were dissected out from the animals, collected in pre-labelled small plastic bags and placed on dry ice. Left sided subcutaneous (SC) and epididymal (Epi) adipose pads, as well as entire mesenteric (Mes) fat depots, were removed from the animals and wrapped in pre-labelled aluminium foil, then snap frozen on dry ice. Left adrenals were removed from the mice and immediately placed into 10% formalin. Twenty-four hours post fixation, adrenals were cleaned of any attached fat by manual dissection, and then weighed on a microbalance. RNA was extracted from liver as described in (2.2.7). Due to an ongoing pinworm infection treatment in the Little France Biomedical Research Facility, Edinburgh, this experiment was carried out in the Hugh Robson Building Animal Unit, Edinburgh, U.K. This dietary experiment is described in Chapter 4 of this thesis.

A second dietary experiment is described in chapter 6 of this thesis. Male, adult (8 week old) LKO, littermate *Hsd11b1*$^{flox/flox}$ control, Del, and HSD1 KO mice (n = 7-10/group) were fed high fat diet (D12331; Research Diets Inc., U.S.A.) for 14 weeks. Body weight was measured weekly and glucose tolerance test (GTT) carried out at week 13 of the diet (see 2.2.4). Mice were culled by cervical dislocation and blood was collected in EDTA tubes for plasma collection. Liver, white adipose tissue depots (left SC, left Epi, whole Mes), left quadriceps muscle, and left adrenal glands were harvested as described above. Liver, white adipose tissue depots and muscle were quickly weighed before being snap frozen on dry ice, whereas adrenals were fixed in 10% formalin for 24 h before being weighed as described above. Right sided SC fat depots were also collected in 10% formalin for picrosirius red staining (see 2.2.15). RNA was extracted from liver as described in (2.2.7).

Table 2. 5 Chow diet constituents

Chow Diet (Product code 801722, Special Diets Services)	**kcal%**
Protein	22.0
Carbohydrate	68.9
Fat	9.1
Ingredients	**(%)**
Crude Oil	3.05
Crude Protein	16.44
Total Dietary Fibre	15.06
Pectin	1.40
Hemicellulose	8.85
Cellulose	3.89
Lignin	1.40
Starch	42.37
Sugar	3.90
Saturated Fatty Acids	**(%)**
C12:0 Lauric	0.03
C14:0 Myristic	0.14
C16:0 Palmitic	0.33
C18:0 Stearic	0.06
Monounsaturated Fatty Acids	**(%)**
C14:1 Myristoleic	0.02
C16:1 Palmitoleic	0.10
C18:1 Oleic	0.87
Polyunsaturated Fatty Acids	**(%)**
C18:2(ω6) Linoleic	0.96
C18:3(ω3) Linolenic	0.11
C20:4(ω6) Arachidonic	0.11
Amino Acids	**(%)**
Arginine	1.19
Lysine	1.04
Methionine	0.28
Cystine	0.29
Tryptophan	0.22
Histidine	0.46
Threonine	0.69
Isoleucine	0.77
Leucine	1.46
Phenylalanine	0.96
Valine	0.91
Tyrosine	0.69
Glycine	1.55

Aspartic Acid	1.00
Glutamic Acid	3.72
Proline	1.34
Serine	0.78
Alanine	0.21
Macro Minerals	**(%)**
Calcium	0.83
Total Phosphorus	0.64
Phytate Phosphorus	0.23
Available Phosphorus	0.41
Sodium	0.27
Chloride	0.40
Potassium	0.69
Magnesium	0.22
Micro Minerals	
Iron	130.65 mg/kg
Copper	16.42 mg/kg
Manganese	91.05 mg/kg
Zinc	86.59 mg/kg
Cobalt	494.92 µg/kg
Iodine	390.43 µg/kg
Selenium	265.49 µg/kg
Fluorine	9.63 mg/kg
Vitamins	
β-Carotene	1.28 mg/kg
Retinol	5218.35 µg/kg
Vitamin A	17376.38 iu/kg
Cholecalciferol	76.94 µg/kg
Vitamin D	3077.42 iu/kg
α-Tocopherol	93.03 mg/kg
Vitamin E	102.81 iu/kg
Vitamin B_1 (Thiamine)	15.84 mg/kg
Vitamin B_2 (Riboflavin)	13.28 mg/kg
Vitamin B_6 (Pyridoxine)	17.65 mg/kg
Vitamin B_{12} (Cyanocobalamine)	78.17 µg/kg
Vitamin C (Ascorbic Acid)	1.80 mg/kg
Vitamin K	185.05 mg/kg
Folic Acid (Vitamin B_9)	4.30 mg/kg
Nicotinic Acid (Vitamin PP)	78.92 mg/kg
Pantothenic Acid (Vitamin $B_{3/5}$)	25.24 mg/kg
Choline (Vitamin $B_{4/7}$)	899.51 mg/kg
Inositol	2253.88 mg/kg
Biotin (Vitamin H)	488.74 µg/kg

[Table adapted from manufacturer's diet sheet]

Table 2. 6 High fat diet constituents

High Fat Diet (Product #D12331, Research Diets Inc.)	gm%	kcal%
Protein	23.0	16.4
Carbohydrate	35.5	25.5
Fat	35.8	58
Ingredients	**gm**	**kcal**
Caesin, 30 Mesh	228	912
DL-Methionine	2	0
Maltodextrin 10	170	680
Corn Starch	0	0
Sucrose	175	700
Soybean Oil	25	225
Coconut Oil, Hydrogenated	333.5	3001.5
Mineral Mix S10001	40	0
Sodium Bicarbonate	10.5	0
Potassium Citrate, 1 H2O	4	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
FD&C Red Dye #4	0.1	0
Total	**1000.1**	**5558.5**

[Table adapted from manufacturer's diet sheet]

Table 2. 7 Western diet constituents

Western Diet (Product #D12079B, Research Diets Inc.)	**gm%**	**kcal%**
Protein	20	17
Carbohydrate	50	43
Fat	21	41
Ingredients	**gm**	**kcal**
Caesin, 80 Mesh	195	780
DL-Methionine	3	12
Corn Starch	50	200
Maltodextrin 10	100	400
Sucrose	341	1364
Cellulose	50	0
Milk Fat, Anhydrous*	200	1800
Corn Oil	10	90
Mineral Mix S10001	35	0
Calcium Carbonate	4	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
Cholesterol, USP*	1.5	0
Ethoxyquin	0.04	0
Total	**1001.54**	**4686**

* Anhydrous milk fat typically contains approximately 0.3% cholesterol. On this basis, D12079B contains approximately 0.21% cholesterol.

[Table adapted from manufacturer's diet sheet]

2.2.4. Glucose Tolerance Test (GTT)

Mice were housed individually for several days before carrying out GTT, so that they could acclimatise to single-housing. Mice were fasted for 6 h, and then blood was taken for plasma glucose assay. Initially, 10µl blood was taken from a tail nick, into EDTA-coated microtubes (Sarstedt, U.K.). Mice were then injected intraperitoneally with 2mg glucose/g body weight. Further blood samples (10µl/sample) were taken at 15 min, 30 min, 60 min, and 120 min after the injection. Blood was centrifuged at 2500g, 4°C for 10 min, plasma extracted and stored at -80°C for later analysis.

2.2.5. Plasma Glucose Measurement

Plasma glucose levels were quantified using an Infinity Glucose Hexokinase Liquid Stable Reagent (Thermo Electron, U.S.A.). A series of glucose standards ranging from 0-400mg/dL was used to produce a standard curve. In a 96-well plate, 2µl of standard or sample plasma were mixed with 250µl of glucose hexokinase reagent and incubated at room temperature, in the dark, for 3 min. The reagent causes the glucose within the sample to be phosphorylated, generating NADH. The absorbance of NADH within the plate was then measured at 340nm using a plate-reader spectrophotometer (Molecular Devices, OPTImax). The glucose concentration within the plasma samples was determined with reference to the standard curve.

For the dietary experiment described in Chapter 6, blood glucose levels were also measured using a handheld glucometer (OneTouch Ultra, LifeScan, U.S.A.) on a drop of tail-vein blood, according to the manufacturer's protocol.

2.2.6. Plasma Insulin Measurement

Plasma insulin levels were measured using a Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem, U.S.A.). An insulin standard curve (0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6 ng/ml) was made using the mouse insulin stock solution supplied with the kit. In a 96-well plate, 5µl of standards or sample plasma were mixed with 95µl of sample diluent buffer (supplied with the kit) and incubated at 4°C for 2 h. The

solution was aspirated and the wells washed 5 times with washing buffer (supplied with the kit; 300µl per well). Into each well, 100µl of anti-insulin enzyme conjugate solution (supplied with the kit) was then added. After incubation for 30 min at room temperature, the wells were washed 7 times as described above. Then, 100µl enzyme substrate solution (supplied with the kit) was added into each well, and after 40 min incubation at room temperature, without exposure to light, the reaction was stopped by adding 100µl of stop solution (supplied with the kit) to each well. The plate was read within 30 min, using a plate-reader spectrophotometer (Molecular Devices, OPTImax), at two wavelengths- 450nm and 630nm. The reading subtraction results (A450-A630) were compared to the standard curve to calculate the sample concentrations.

2.2.7. RNA Extraction from Tissue

Total RNA was extracted from liver tissue using TRIzol® reagent (Invitrogen, U.K.). Prior to RNA extraction, in order to protect RNA samples from RNase contamination, all the apparatus used was autoclaved and all solutions prepared with DEPC-treated/RNase-free water.

Snap-frozen liver samples were weighed and ~50mg homogenized in 1000µl of TRIzol® reagent. The homogenate was either used immediately or frozen at -80°C for later extraction. To extract RNA, 200µl of chloroform was added to each sample, which was then vigorously shaken for 15-30 s. The samples were then incubated at room temperature for 2-3 min, after which they were centrifuged at 12,000g, 4°C for 15 min. The upper (aqueous) phase was transferred to a fresh tube, mixed with 500µl isopropanol, and allowed to sit at room temperature for 10 min. Samples were then centrifuged at 12,000g, 4°C for 10 min. After this centrifugation step, the supernatant was discarded, and the pellet washed with cold 50-75% ethanol, vortexed for 15 s and centrifuged at 7500g, 4°C for 10 min. Once again, the supernatant was discarded. The RNA pellet was allowed to air dry before finally resuspending in 30-50µl RNase-free water. RNA was processed immediately or stored at -80°C.

2.2.8. RNA Quantification and Quality Control

RNA concentration was measured using a NanoDrop spectrophotometer at a wavelength of 260nm. RNA eluate (1.2µl) was placed on the NanoDrop platform, and the values for RNA concentration, 260/280nm and 230/260nm ratios were recorded. RNA integrity was routinely analysed by gel electrophoresis in 1% agarose either in 0.5x TBE or under denaturing conditions (see 2.2.9). The quality of RNA was assessed by the presence of two distinct bands, corresponding to 28S and 18S rRNA at the approximate intensity ratio of 2:1, with no additional smears or high-molecular weight genomic DNA contamination (Figure 2.3).

Additionally, some RNA samples were analysed using an Agilent 2100 Bioanalyzer. Gel-dye solution was prepared by mixing 65µl of gel matrix (supplied with the kit) with 1µl of dye (supplied with the kit) and centrifuged at 13,000g, room temperature, for 10 min. The chip was placed into the priming station and 9µl of the gel-dye mix was placed in the well marked "G". The chip priming station was closed, plunger positioned at 0.7ml and pressed. After 5 min, the plunger was pulled back and 5µl of the sample marker was placed in each well of the chip. Ladder and RNA samples (1µl) were then loaded onto the chip, vortexed and placed in the analyser. Good quality total RNA was characterised by two distinct 18S and 28S peaks, and an RNA Integrity Number (RIN; incorporating the entire electrophoretic trace analysis) higher than 9.

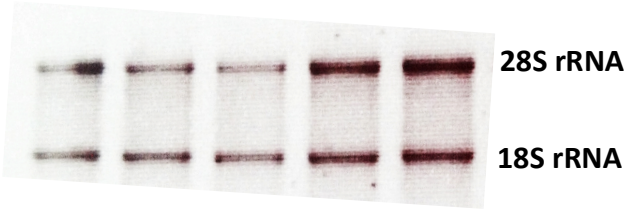


Figure 2.3: Representative RNA agarose gel image.

Distinct 28S and 18S bands can be seen (with roughly a 2:1 ratio, respectively).

2.2.9. Agarose Gel Electrophoresis

Agarose gels were prepared with, and electrophoresed in, 0.5x TBE buffer. For genotyping PCR products, a 1.5% agarose gel was used, and for primer specificity testing, a 3.5% agarose gel was used.

Agarose was melted in the TBE buffer using a microwave. GelRed Nucleic Acid Stain (0.005% final concentration) was added to molten agarose before pouring into a gel tray. Samples (excluding PCR samples already containing loading concentrate) were mixed with Orange G loading dye (2:10) and loaded onto the gel. A DNA ladder was loaded into one lane.

Denaturing gels (1.2% agarose) were prepared in MOPS buffer with 0.8% formaldehyde. RNA samples were mixed with 400U/ml RNasin® Plus RNase inhibitor, 10mM DTT and DEPC-treated water in a final volume of 10µl. 2.5µl of 37% formaldehyde, 2.5µl of 10x MOPS and 10µl of deionised formamide were added. Samples were then heated at 65°C for 15 min, quickly chilled and mixed with Orange G loading buffer before electrophoresis on a denaturing gel in 1x MOPS buffer.

Gels were electrophoresed at ~80-130V and visualised under UV light (λ=260nm) on a transilluminator.

2.2.10. Quantitative PCR

2.2.10.1. cDNA Synthesis

Reverse transcription of RNA was carried out using the SuperScript® III reverse transcriptase system (Invitrogen, UK). Each 20µl reaction used 1µg of total RNA. In order to remove all traces of genomic DNA contamination prior to first-strand cDNA synthesis, 1µg of all RNA samples were initially treated with DNase I (Invitrogen, UK) according to the manufacturer's protocol. RNA was then denatured for 5 min at 65°C with 25ng random primers and dNTPs at 0.5mM final concentration. Tubes were immediately placed on ice for 1 min, then briefly centrifuged to collect the

contents. To each sample, 4µl of 5x First Strand buffer, 1µl 0.1mM DTT, 1µl SuperScript® reverse transcriptase and 1µl RNasin® Plus RNase inhibitor were added. Reactions were incubated at 25°C for 5 min, followed by 50°C for 60 min and 70°C for 15 min. Control reactions with no enzyme or no RNA were included to detect contamination of reagents. cDNA was stored at -20°C for further analysis.

2.2.10.2. Quantitative (q, real-time) PCR

Quantitative PCR (qPCR) was used to measure mRNA levels in tissues. Reactions were carried out in a LightCycler® 480 thermocycler (Roche Diagnostics Ltd., UK) using 480 Probes Master Mix and fluorescent probes from the Universal Probe Library (UPL; Roche Diagnostics Ltd., UK). Primer sets were designed with ProbeFinder software within the UPL system, so as to span exon-exon junctions in the target mRNA to prevent amplification of the target from any contaminating genomic DNA. Each custom primer set was tested for the presence of one product of expected size by gel electrophoresis (see 2.2.9). Details of each assay can be found in Table 2.8.

A standard curve was prepared by serially diluting a mix of equal volumes of all cDNA samples (standard curve range was from 1 to 1:256 for all gene expression assays). cDNA samples were diluted in sterile water prior to assay (1:20 for liver and 1:10 for adipose tissue). The reaction mix was prepared as follows: 5µl LightCycler 480 Probes Master Mix, 0.1µl probe (final concentration 1pmol), 0.1µl of each primer (final concentration 0.2pmol), 2.7µl sterile water per sample. Reaction mix was added to a 384-well plate, followed by 2µl of cDNA sample/standards. Each sample was analysed in triplicate. Plates were sealed and centrifuged (2000g, room temperature, 2 min) to ensure all solutions were in the wells. The relative amplification levels for each sample were measured. Reaction conditions were as follows: 95^{o}C for 5 min; 50 cycles of 95^{o}C for 10 s, 60^{o}C for 30 s, 72^{o}C for 1 s; cooling 40^{o}C for 30 s.

Data were analysed using the 2nd derivative maximum method, where a fractional cycle (Cp) is determined from the amplification curve's second derivative max indicating the cycle at which exponential amplification can no longer be sustained and

Table 2. 8 List of qPCR primer sequences and probe numbers

Gene of Interest	Primers	UPL Probe #
18S	Forward: ctcaacacgggaaacctcac Reverse: cgctccaccaactaagaacg	77
Actb	Forward: ctaaggccaaccgtgaaaag Reverse: accagaggcatacagggaca	64
Hprt	Forward: tcctcctcagaccgctttt Reverse: cctggttcatcatcgctaatc	95
Tbp	Forward: gggagaatcatggaccagaa Reverse: gatgggaattccaggagtca	97
Hsd11b1	Forward: tctacaaatgaagagttcagaccag Reverse: gccccagtgacaatcacttt	1
Srebp2	Forward: cagacagccaatggcaca Reverse: atctgaggttgcaccaggac	62
Hmgcr	Forward: tgattggagttggcaccat Reverse: tggccaacactgacatgc	78
Hmgcs	Forward: cagggtctgatccccttg Reverse: cagagaactgtggtctccaggt	41
Lxrα	Forward: tgtgcgctcagctcttgt Reverse: tggagccctggacattacc	71
Srebp1c	Forward: ttcctcagactgtaggcaaatct Reverse: agcctcagtttacccactcct	74

Gene of Interest	Primers	UPL Probe #
Cyp7a1	Forward: acaccattcctgcaaccttc Reverse: tcttggccagcactctgtaa	92
Abcg5	Forward: tcctgcatgtgtcctacagc Reverse: atttgcctgtcccacttctg	31
Abcg8	Forward: aagacgggctgtacactgct Reverse: ggcatcgcgtagatgatga	98
Abca1	Forward: gcagatcaagcatcccaact Reverse: ccagagaatgtttcattgtcca	1
Apoe	Forward: gaccctggaggctaaggact Reverse: agagccttcatcttcgcaat	12
ApoA1	Forward: tatgtggatgcggtcaaaga Reverse: tgaacccagagtgtcccagt	63
Cyp7b1	Forward: gcttggtctgcctggaaa Reverse: acttctcggatgatgctgga	99
Cyp27a1	Forward: atgggatcttcatcgcaca Reverse: cgtttaaggcatccgtgtaga	66
Abcg1	Forward: gggtctgaactgccctacct Reverse: tactcccctgatgccacttc	3

starts to decline towards linear growth. The efficiency of each assay was ~2. The standard curve was used as a reference for extrapolating quantitative information for mRNA targets of unknown concentrations.

2.2.10.2.1. Validation of internal controls

Gene expression levels were determined by normalising mRNA levels of the gene of interest to the levels of an internal standard mRNA. In preliminary experiments, based on the availability within the lab, *Actb*, *Hprt* and *Tbp* were tested to confirm their relevance as internal standards (housekeeping genes). These candidate internal controls had similar Cp values and cycle-amplification profile between experimental groups; their mRNA levels did not change with genotype or dietary manipulation (Figure 2.4) thereby validating their use as internal controls. The specific internal control(s) used in each experiment can be found in Chapters 3-6.

2.2.11. Protein Extraction and Analysis

2.2.11.1. Protein Extraction from tissues

Total protein was extracted from frozen tissue; 50-100mg of frozen tissue samples were homogenised in 500µl lysis buffer and incubated for 1 h, 4°C with rotation before centrifugation at 12,000g, 4°C for 5 min. The supernatant was then transferred into a fresh tube and processed immediately or stored at -20°C.

2.2.11.2. Protein Quantification

Protein concentration of lysates was determined using the Bio-Rad D_C protein assay (Bio-Rad). This is a colourimetric assay for protein determination. Sample concentrations were determined from a standard curve created from a series of dilutions of bovine serum albumin (BSA), ranging from 0-1.2mg/ml. To ensure all samples lay within the standard curve, a test assay was carried out using the most diluted sample within the set. This sample was compared with the highest protein standard (1.2mg/ml) using the Bio-Rad protein assay. Dilution was required if the

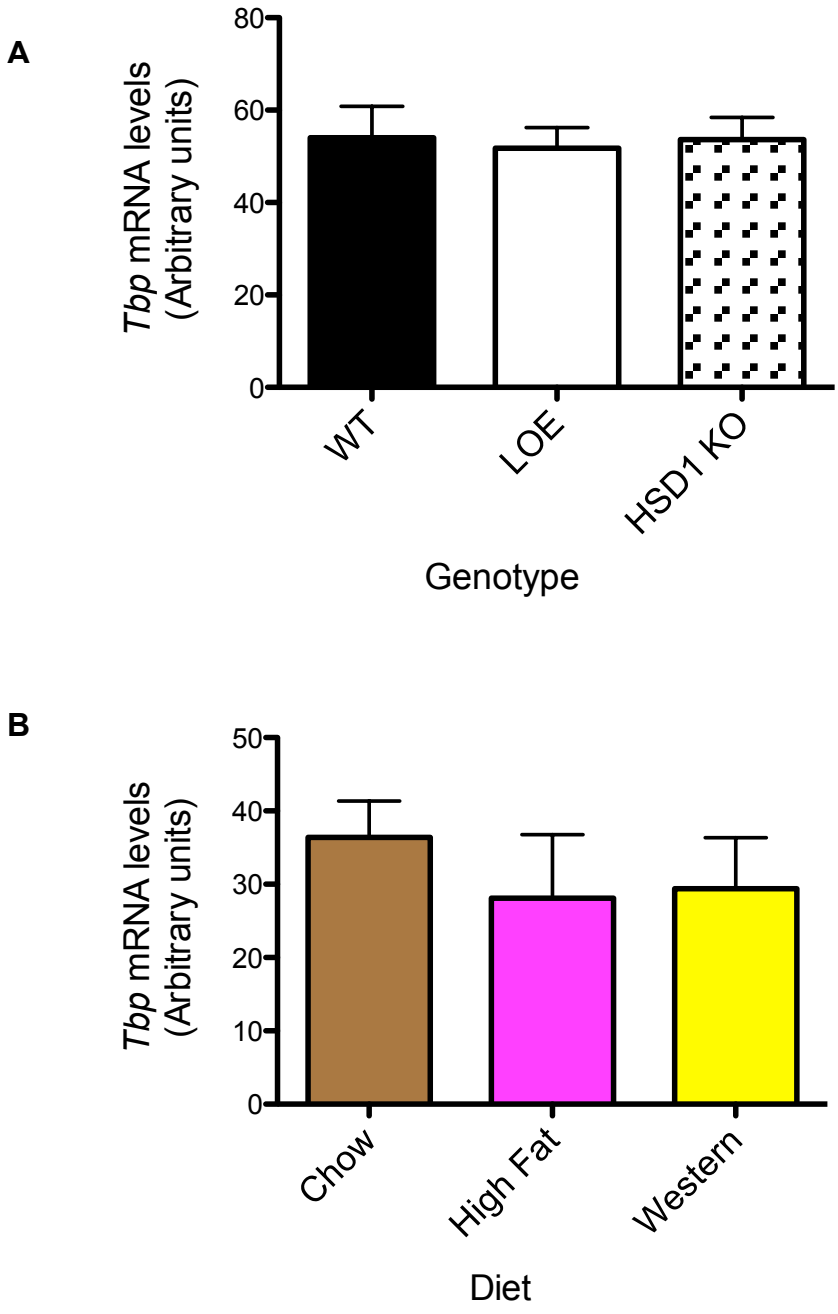


Figure 2.4: Validation of internal controls

Representative experiments demonstrating lack of differences in hepatic *Tbp* mRNA levels with **(A)** genotype or **(B)** diet.

sample shared a similar or greater colour to the highest standard. All samples were diluted according to the test assay and measured in duplicate in a 96-well plate. To each well, 5µl of diluted sample or standard was added followed by 25µl of reagent A' (5% v/v reagent S in reagent A), and 200µl of reagent B. The plate was then incubated at room temperature for 15 min before reading in a plate-reader spectrophotometer at 750nm (Molecular Device, OPTImax). A standard curve was created by measurement of absorbance of the series of concentrations of BSA. Absorbance values of unknown samples were interpolated onto the standard curve to determine their concentrations.

2.2.11.3. Separation of Proteins by Electrophoresis (SDS-PAGE)

Protein samples (25µg-40µg) were adjusted to 6.5µl with water and added to a pre-prepared mix of 2.5µl NuPAGE® SDS sample buffer (Invitrogen, UK) and 1µl of NuPAGE® reducing agent (Invitrogen). Samples were incubated at 70^{o}C for 10 min to denature the protein. Samples and 5µl of Full-Range Rainbow marker (Invitrogen, UK) were loaded onto a 4-12% Bris-Tris Novex® pre-cast gel (Invitrogen, UK). The inner chamber of the electrophoresis tank was filled with 200ml of 1x NuPAGE® MES running buffer (Invitrogen, UK) and 500µl of NuPAGE® antioxidant (Invitrogen, UK), and the outer chamber with 600ml of 1x NuPAGE® MES running buffer. Samples were electrophoresed at 200V on ice until the dye front reached the bottom of the gel.

2.2.11.4. Coomassie Gel Staining

Following electrophoresis, the gel tank was disassembled and the gel transferred into a dish containing Coomassie staining solution and agitated gently overnight until uniformly blue. The gel was then placed in destain solution (45% v/v methanol, 10% v/v acetic acid) and agitated as above. The solution was changed, when appropriate, until the gel became translucent with visible protein bands and no further blue dye transferred into the destain solution.

2.2.11.5. Western blot

Following electrophoresis, the gel tank was disassembled, and the gel transferred into a transfer sandwich (from the bottom: sponge-paper-gel-membrane-paper-sponge). Sponges were pre-soaked for 1 h in cold (4°C) transfer buffer. The sandwich cassette was then placed in the tank and all chambers filled with transfer buffer. Proteins were transferred at 30V on ice for 60 min. Following the transfer, the membrane was placed into the Ponceau Red solution (see 2.2.11.6) or directly into blocking buffer and incubated under agitation for 1 h at room temperature. The blocking solution was then removed and the membrane incubated with primary antibody diluted into blocking buffer. Antibody dilutions were as follows: rabbit polyclonal anti-SREBP-2 1:500, sheep polyclonal anti-11β-HSD1 (raised in sheep, kind gift from Scott Webster; De Sousa Peixoto *et al*., 2008; Turban *et al*., 2012; Coutinho *et al*., 2013) 1:1000, mouse polyclonal β-tubulin 1:10,000. The membrane was incubated overnight at 4°C under agitation, and then washed three times (10 min/wash) in TBST buffer, at room temperature under agitation. The membrane was then incubated for 1 h at room temperature under agitation in the dark with the appropriate IRdye® secondary antibodies diluted 1:10,000 in blocking solution. Finally, the membrane was rinsed with TBST three times as described above and imaged using a LI-COR® Odyssey infrared scanner (2.2.12.7).

2.2.11.6. Ponceau Red Staining

To assess transfer efficiency, the membrane was placed into a dish containing Ponceau Red solution, swirled for 2 min, then the membrane was washed with water until no more red dye eluted and the protein bands were clearly visible by eye. The membrane was then blocked and processed as above.

2.2.11.7. Analysis and quantification

Protein levels were measured using an infrared LI-COR® Odyssey scanner. Membranes were scanned at two wavelengths, 700nm (red channel) and 800nm (green channel). The protein of interest and internal control were detected and

analysed within the same membrane by drawing equal sized rectangles around each band, with the scanner calculating optical density.

2.2.12. Hepatic Subcellular Fractionation (Hepatic Microsomal Extraction)

Livers were stored at –80°C until use when they were slowly defrosted on wet ice. Each sample was cut into small pieces (100mg) and disrupted repeatedly (15 sec) in glass tubes (10x1cm) containing sucrose buffer (1ml). Tubes were stored on wet ice. Homogenates were then transferred into clean glass tubes (13x1cm), and sucrose buffer was added to 90% tube height. Samples were subjected to centrifugation (1000g, 4°C, 15 min) for separation of cell debris and the nuclear fraction (pellet). The supernatant was transferred into Beckman ultracentrifuge tubes (5x1cm). A further centrifugal separation (13,000g, 4°C, 30 min) was performed. The subsequent supernatant was collected in clean Beckman ultracentrifuge tubes and another centrifugal separation was performed (23,913g, 4°C, 1 h) to obtain light microsomes (pellet) and cytosolic fractions (supernatant). The supernatant was stored at –80°C. The remaining pellet of microsomes was reconstituted in sucrose buffer (500µl). Aliquots (40µl) were removed for immediate quantitation of protein (as in section 2.2.11.2) and the remaining sample stored at –80°C until further use.

2.2.13. Hepatic Nuclei Purification

Livers were stored at –80°C until processing. Each liver sample (~100mg) was homogenised in homogenisation buffer containing 10mM EGTA, 10mM EDTA, 0.25M sucrose, 2mM $MgCl_2$, 20mM Tris-HCL (pH 7.4), and subsequently centrifuged at 2500g for 5 min. Pellets were dissolved in homogenisation buffer and centrifuged at 1000g for 5 min. The supernatant was aspirated and pellets were dissolved in buffer containing 1mM EGTA, 1mM EDTA, 1.5mM $MgCl_2$, 2.5% v/v glycerol and 20mM HEPES KOH (pH 7.6) for 60 min at 4°C. The nuclear extract was obtained after centrifugation at 100,000g, 4°C for 30 min. The protein concentration of each liver nuclear sample was measured as in section 2.2.11.2, and the samples were stored at –80°C until use.

2.2.14. 11β-HSD1 Activity Assay

Liver homogenates (0.1mg/ml) were incubated in duplicate at 37°C with 25nM ^{3}H-corticosterone (Amersham Pharmacia Biotech, U.K.) and an excess (400μM) of the 11β-HSD1-specific cofactor NADP. Under *in vitro* conditions, in homogenates, 11β-HSD1 is bidirectional and the dehydrogenase assay is more reliable. This assay was in the linear range of protein concentration and product formation. Briefly, after a 25-min incubation, steroids were extracted with ethyl acetate, separated by thin-layer chromatography (TLC), identified by migration in comparison to unlabelled standards, exposed to phosphorimager film (FLA2000; Fujifilm, U.K.) for 1-3 days, and analysed by quantitative phosphorimager software (Aida; Raytek Scientific, Sheffield, U.K.).

In another assay, liver homogenates (25μg/ml) were incubated in duplicate at 37°C in Krebs-Ringer buffer containing 0.2% glucose, 2mM NADP, 50nM ^{3}H-corticosterone, and unlabelled corticosterone (0.3-10μM). After 45 min, steroids were extracted with ethyl acetate, the organic phase was evaporated under nitrogen, and extracts were resuspended in mobile phase (60% water, 15% acetonitrile, and 25% methanol). Steroids were separated by high-performance liquid chromatography (HPLC) using a reverse phase μ-Bondapak C18 column (Phenomenex, UK) and were quantified by liquid scintillation counting. Each sample was measured for at least 25 min to ensure that peaks for both corticosterone and 11-dehydrocorticosterone were completely quantified.

2.2.15. Adipose Picrosirius Red Staining

Right sided subcutaneous fat depots excised from high fat diet-fed LKO, control, Del and HSD1 KO mice (Chapter 6; see 2.2.3) were fixed in 10% phosphate-buffered formalin solution for 24 h. Tissues were then transferred to 70% ethanol and embedded in paraffin blocks for subsequent sectioning. Using a microtome, sections of 5μm thickness were processed and collected onto electrostatic slides, which were then incubated at 37°C overnight. Total collagen content in subcutaneous fat was determined by staining the formalin-fixed paraffin sections of subcutaneous adipose

tissue in picrosirius red (Sigma-Aldrich). Slides containing the tissue sections were immersed in picrosirius red solution for 2 h in the dark, after which they were rinsed in water, dehydrated and mounted. Adipose sections from each experimental group were processed simultaneously. The slides were viewed with a Zeiss light microscope. Pictures were acquired using a camera attached to the microscope and ImageJ software was used to quantify picrosirius red staining.

2.2.16. Statistical Analyses

For statistical analysis, GraphPad Prism 5 was used, except for Chapter 4 of this thesis, where data were analysed using GraphPad Prism 6 (allowing the use of Tukey's and Fisher's LSD *post-hoc* tests following two-way ANOVA).

Comparisons between 2 groups (e.g. LOE versus WT) were made by Student's *t*-test (unpaired). When variances between groups were not equal, an unpaired *t*-test with Welch's correction was used. For the analysis of an effect of one factor (e.g. diet) in three or more groups, one-way ANOVA with Tukey's *post-hoc* test was used. For the analysis of multiple levels of two factors (e.g. genotype and diet), two-way ANOVA with Tukey's *post-hoc* test (unless specified otherwise) was used.

Values are shown as means ± SEM. Significance was set as $p<0.05$. Full details of statistical analyses are contained within figure legends in each chapter.

Chapter 3. Effect of Increased Hepatic 11β-HSD1 on Cholesterol Homeostasis

3.1. Introduction

In mice, deficiency (knockout) of 11β-HSD1 is protective against the adverse metabolic consequences of diet-induced obesity such as glucose intolerance and dyslipidaemia (Kotelevtsev *et al*., 1997; Morton *et al*., 2001; Morton *et al*., 2004). More recently, antidiabetic effects of pharmacological 11β-HSD1 inhibition have been observed in mice (Lloyd *et al*., 2009). Furthermore, in type 2 diabetes patients, inhibition of 11β-HSD1 reduces levels of plasma glucose, glycated haemoglobin A1c (HbA1c) and cholesterol (Rosenstock *et al*., 2010). Also, in mouse models, knockout or inhibition of 11β-HSD1 offers atheroprotection, despite only modest or no changes in plasma cholesterol levels (Hermanowski-Vosatka *et al*., 2005; Kipari *et al*., 2013; Luo *et a*l., 2013).

Transgenic mice expressing increased 11β-HSD1 activity selectively in the liver (LOE mice) exhibit insulin resistance, fatty liver (mainly as TG), and dyslipidaemia (Paterson *et al*., 2004). These mice also have elevated hepatic peroxisome proliferator-activated receptor alpha (PPARα) and mitochondrial carnitine palmitoyltransferase 1 (mCPT-1) expression, suggesting activation of lipid oxidation (Paterson *et al*., 2004). The acetyl-CoA produced from lipid oxidation may be utilised for cholesterol synthesis in LOE mice. Despite no differences in serum cholesterol levels or cholesterol distribution profile between LOE and control mice fed low-fat diet, high fat (HF) feeding altered the cholesterol lipoprotein profile in LOE mice, with decreased plasma HDL cholesterol but more cholesterol in low density lipoproteins (Paterson *et al*., 2004). The investigators also observed a trend for increased hepatic HMG-CoA reductase mRNA expression in LOE mice, though this did not achieve significance (Paterson *et al*., 2004). These liver-specific 11β-HSD1 overexpressor mice were first created on a mixed background of C57BL/6xCBA/C3H, and F_3 C57BL/6 backcross male mice were used for the initial study (Paterson *et al*., 2004). Since then, these mice have been backcrossed >10 generations and, at the start of this PhD study, this model was already congenic on the C57BL/6 background.

Apart from catalysing the intracellular conversion of GCs, 11β-HSD1 also catalyses reduction of 7-KC to 7-βHC (Schweizer *et al*., 2004; Hult *et al*., 2004). 7-KC has the potential to regulate cholesterol biosynthesis because it is an inhibitor of SREBP-2 cleavage (Brown *et al*., 2002). In liver microsomes of LOE mice, levels of 7-KC are significantly reduced compared to levels in control mice (Dr. T. Mitic, unpublished data; Figure 3.1). Furthermore, pilot data obtained in collaboration with Dr. T. Mitic at the beginning of this study revealed that liver microsomes from LOE mice have increased levels of cleaved (mature) SREBP-2 protein compared to wild-type controls (Figure 3.2). Because mature SREBP-2 transcriptionally activates genes in the cholesterol biosynthetic pathway (Horton *et al*., 1998), this finding suggests increased cholesterol synthesis in LOE mice.

Liver X receptors (LXR) are sterol sensors and their role in protecting cells from cholesterol overload by stimulating reverse cholesterol transport (RCT) and activating its conversion to bile acids in the liver is well established (Zhang and Mangelsdorf, 2002; see 1.5.5). A common connection between SREBP-2 and LXR-mediated processes is their regulatory response to certain oxysterols. Whilst certain oxysterols inhibit SREBP-2 activation, they serve as ligands for LXR (Willy *et al*., 1995; Teboul *et al*., 1995; Peet *et al*., 1998; Venkateswaran *et al*., 2000; DeBose-Boyd *et al*., 2001; Janowski et al., 2001). As a transcription factor with a key role in regulating cholesterol biosynthesis, SREBP-2 may positively regulate LXR-target genes by enabling the generation of oxysterol ligands for LXR. This, indeed, has been implied as well as observed in multiple studies (Forman *et al*., 1997; Wong *et al*., 2004; Wong *et al*., 2006), suggesting that SREBP-2, which is well-known for its regulatory role in cholesterol synthesis, may also play an indirect role in regulation of LXR through generation of its endogenous ligands. In LOE mice, Paterson *et al*. (2004) observed increased hepatic expression of *Lxrα* (Paterson *et al*., 2004), the isoform that plays dominant roles in RCT and whole-body cholesterol homeostasis (Zhang *et al*., 2012; Hong *et al*., 2012).

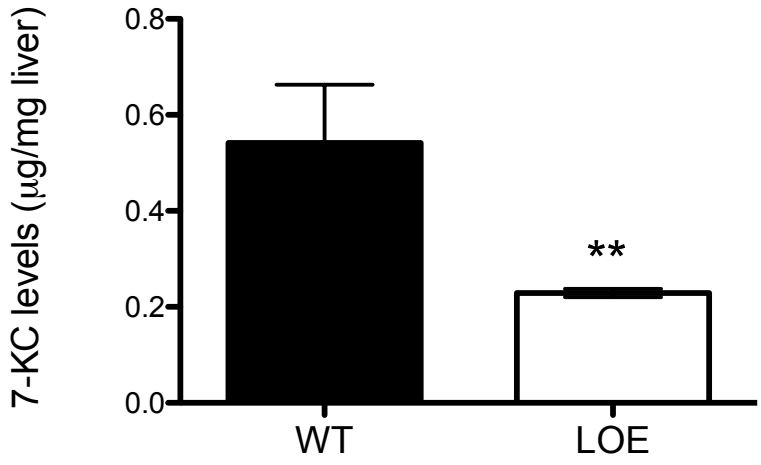


Figure 3.1: Liver microsomes of LOE mice have reduced levels of 7-KC compared to wild-type C57BL/6 mice.

7-Ketocholesterol (7-KC) levels were measured in liver microsomes from chow-fed, 6-8 week old, male wild-type control (C57BL/6) and liver 11β-HSD1 overexpressor (LOE) mice. Values are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=4-6/group; ** $p<0.01$ (Dr. T. Mitic, unpublished data).

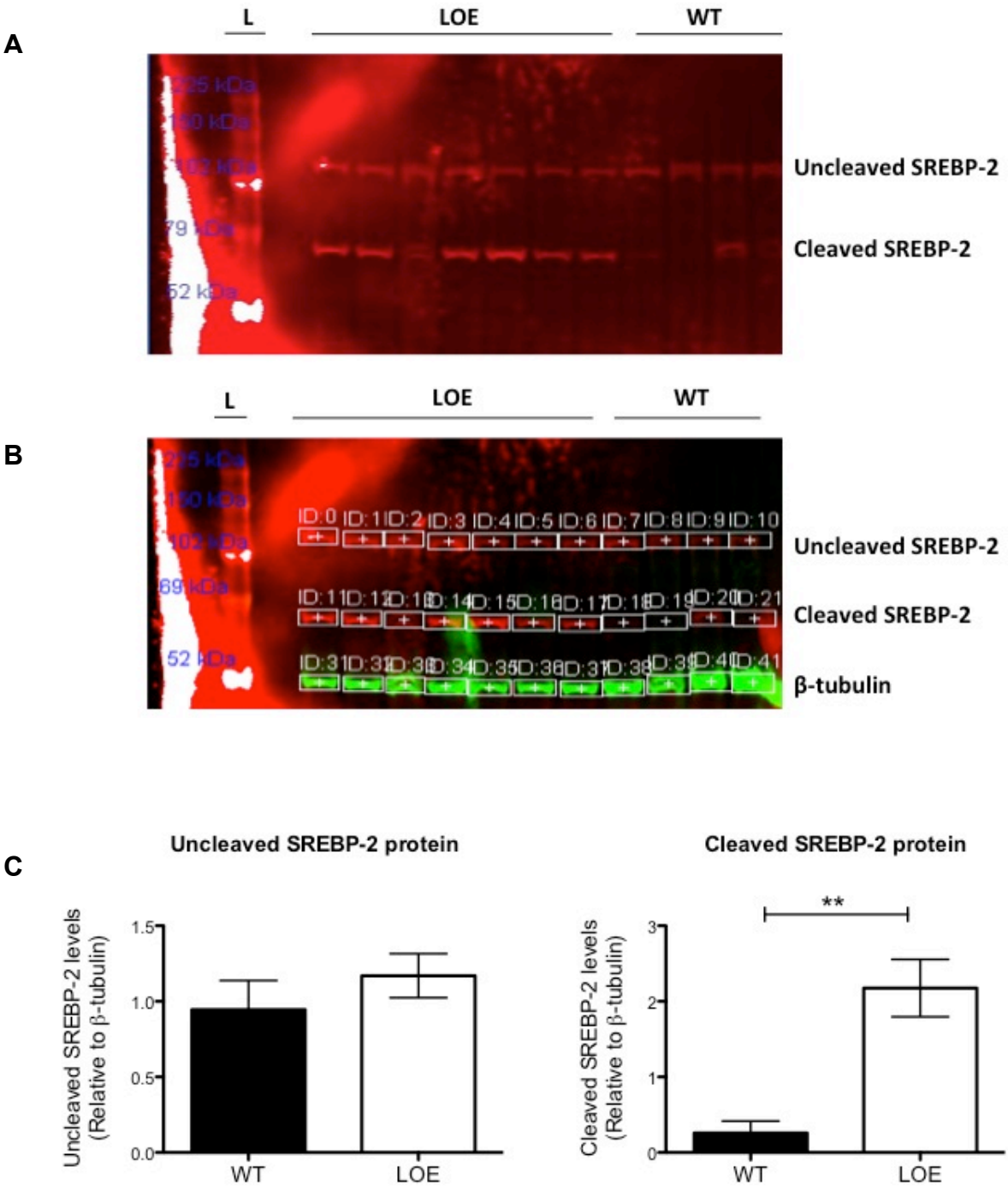


Figure 3.2: Liver microsomes from LOE mice have increased levels of cleaved SREBP-2 compared to wild-type C57BL/6 mice.

Western blot analysis was used to measure levels of uncleaved and cleaved SREBP-2 protein in hepatic microsomes from chow-fed, 6-8 week old, male wild-type (WT) C57BL/6 and liver 11β-HSD1 overexpressor (LOE) mice. **(A)** A representative image of a SREBP-2 western blot, with protein bands for uncleaved as well as cleaved SREBP-2. Each lane contained 25µg of protein from one mouse. **(B)** SREBP-2 protein levels (red) were expressed relative to β-tubulin protein levels (green), the internal control. **(C)** The bands in Figure 3.2B were quantified. Data are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=4-7/group; ** $p<0.01$. L=protein marker/ladder.

The evidence presented above suggests that 11β-HSD1 may play an important role in cholesterol homeostasis. However, this area has been largely unexplored. Liver is the major controller of whole-body cholesterol homeostasis, with major roles in cholesterol biosynthesis, cholesterol uptake through LDLR, lipoprotein release into the blood, storage of cholesterol by esterification, cholesterol degradation (conversion to bile acids), and cholesterol export into bile (Trapani *et al*., 2012; see 1.5.5). Because liver is the principal organ involved in cholesterol homeostasis and the organ with highest expression of 11β-HSD1, the liver enzyme was chosen for study.

To dissect the role of hepatic 11β-HSD1 in cholesterol homeostasis, mouse models with altered levels of 11β-HSD1 selectively in the liver were used to examine effects on hepatic cholesterol homeostasis. LOE mice, with hepatic overexpression of 11β-HSD1 (Paterson *et al*., 2004), provided a valuable resource to study the role of *increased* hepatic 11β-HSD1 in cholesterol homeostasis. As mentioned above, these mice show an adverse metabolic phenotype of fatty liver and dyslipidaemia and, in response to HF diet, display altered cholesterol lipoprotein profile as well as attenuated hepatic induction of cholesterol homeostasis regulators including LXRα (Paterson *et al*., 2004). Furthermore, as mentioned earlier, preliminary data (Figures 3.1 and 3.2) at the start of this study suggested increased cholesterol synthesis in LOE mice. Ideally, liver 11β-HSD1 overexpressors need to be compared to liver-specific 11β-HSD1 knockout mice. Based on data from LOE mice, and according to the hypothesis of this chapter (see 3.2), increased 7-KC and reduced mature SREBP-2 levels are predicted in liver of mice with knockout of hepatic 11β-HSD1, suggesting decreased hepatic cholesterol synthesis. Mice with knockout of liver 11β-HSD1 were generated during the course of this study (Chapter 5). However, the initial experiments focussed on liver 11β-HSD1 overexpressors and wild-type C57BL/6 mice.

3.2. Hypothesis and Aims

As an inhibitor of SREBP-2 cleavage, 7-KC provides an endogenous brake on cholesterol biosynthesis. Therefore, reduced 7-KC levels in liver of LOE mice are predicted to increase hepatic cholesterol synthesis.

Hypothesis: Increased liver 11β-HSD1 promotes *de novo* hepatic cholesterol biosynthesis via increased cleavage of hepatic SREBP-2 and induction of SREBP-2 target genes in the cholesterol biosynthetic pathway.

Aims

1) To test whether hepatic overexpression of 11β-HSD1 increases levels of mRNAs encoding hepatic cholesterol biosynthetic enzymes.

2) To determine whether overexpression of liver 11β-HSD1 leads to activation of hepatic *Lxrα* and its downstream targets involved in cholesterol efflux and removal, in response to excess cholesterol due to increased cholesterol biosynthesis.

3) To confirm that hepatic 11β-HSD1 overexpression leads to increased levels of cleaved SREBP-2 in the nucleus.

3.3. Results

3.3.1. No differences in hepatic *Hmgcr* and *Hmgcs* mRNA levels between wild-type control and LOE mice

Increased cholesterol synthesis is accompanied by an increase in the mRNA levels and activity of key enzymes in the mevalonate pathway. HMG-CoA synthase (HMGCS) catalyses the initial step in this pathway to produce HMG-CoA (Clinkenbeard *et al*., 1975). The enzyme HMG-CoA reductase (HMGCR) then catalyses the conversion of HMG-CoA to mevalonate, the synthesis of which is the committed and rate-determining step in cholesterol formation (Brown and Goldstein, 1980; Goldstein and Brown, 1990). Transcription of these enzymes is regulated by sterol levels, via SREBP-2 (Smith *et al*., 1988; Jiang *et al*., 1993; Vallett *et al*., 1996).

Because preliminary data suggested increased cholesterol biosynthesis in LOE mice (Figures 3.1 and 3.2; see 3.1), SREBP-2 target genes in the cholesterol biosynthetic pathway were examined in livers from 6-8 week old wild-type (WT) C57BL/6 and liver-specific 11β-HSD1 overexpressor (LOE) mice, which were fed a standard chow diet. Messenger RNAs for HMG-CoA reductase as well as for HMG-CoA synthase were measured via qPCR.

Contrary to predictions of the hypothesis, there were no differences in the hepatic levels of *Hmgcr* and *Hmgcs* mRNA between genotypes (Figure 3.3), suggesting no difference in cholesterol synthesis between WT and LOE mice.

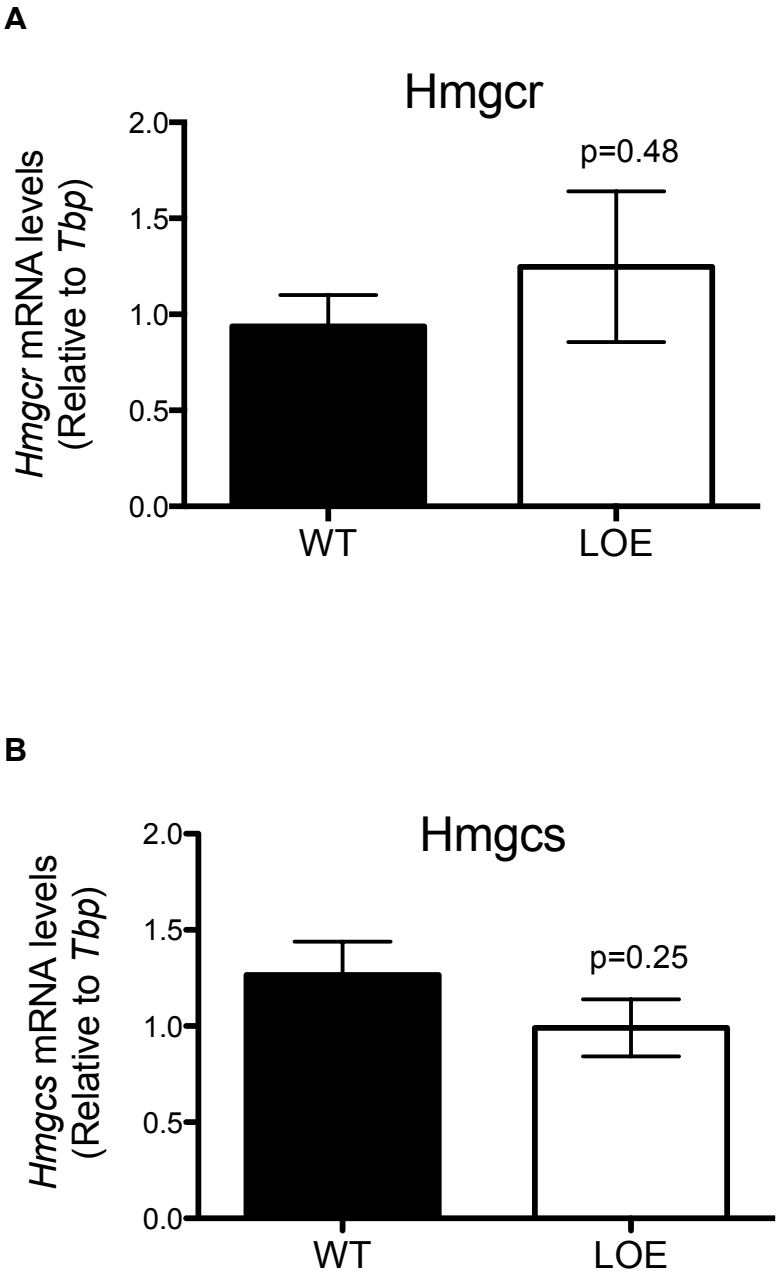


Figure 3.3: Levels of hepatic *Hmgcr* and *Hmgcs* mRNA do not differ between wild-type control and LOE mice.

Total RNA was extracted from liver of chow-fed, 6-8 week old, male wild-type (WT) and liver 11β-HSD1 overexpressor (LOE) mice. Messenger RNA levels were measured by qPCR. **(A)** *Hmgcr* and **(B)** *Hmgcs* mRNA levels are expressed relative to *Tbp* mRNA. Values are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=6/group.

3.3.2. Increased hepatic levels of *Lxrα* mRNA in LOE mice compared to wild-type control mice

The LXR members of the nuclear receptor family are activated by oxysterols such as 22(S)-hydroxycholesterol, 24(S), 25-epoxycholesterol, and 27-hydroxycholesterol, the levels of which are thought to be proportional to cellular cholesterol levels (Janowski *et al*., 1999; Fu *et al*., 2001). Extensive studies have established LXRα as a central regulator of cholesterol homeostasis which regulates a network of genes involved in cholesterol metabolism and excretion (Lehmann *et al*., 1997; Peet *et al*., 1998; Venkateswaran *et al*., 2000; Kennedy *et al*., 2001; Repa *et al*., 2002; Joseph *et al*., 2002; Tangirala *et al*., 2002; Bełtowski, 2008; Hong *et al*., 2012; Zhang *et al*., 2012; see 1.5.5). In addition to cholesterol metabolism and efflux, LXRα activation in animal models increases hepatic fatty acid synthesis (Peet *et al*., 1998; Repa *et al*., 2000a; Schultz *et al*., 2000; Schuster *et al*., 2002; Kalaany *et al*., 2005; Lund *et al*., 2006). Studies have indicated that SREBP-2, the key regulator of cholesterol biosynthesis, upregulates LXR activity through generation of its endogenous ligands (Forman *et al*., 1997; Wong *et al*., 2004; Wong *et al*., 2006).

To investigate if overexpression of liver 11β-HSD1 leads to increased hepatic *Lxrα* expression (in response to excess cholesterol; see 3.2), *Lxrα* mRNA levels were measured in liver from chow-fed, 6-8 week old WT and LOE mice. An increase in hepatic *Lxrα* mRNA levels was observed in LOE mice compared to WT controls (Figure 3.4).

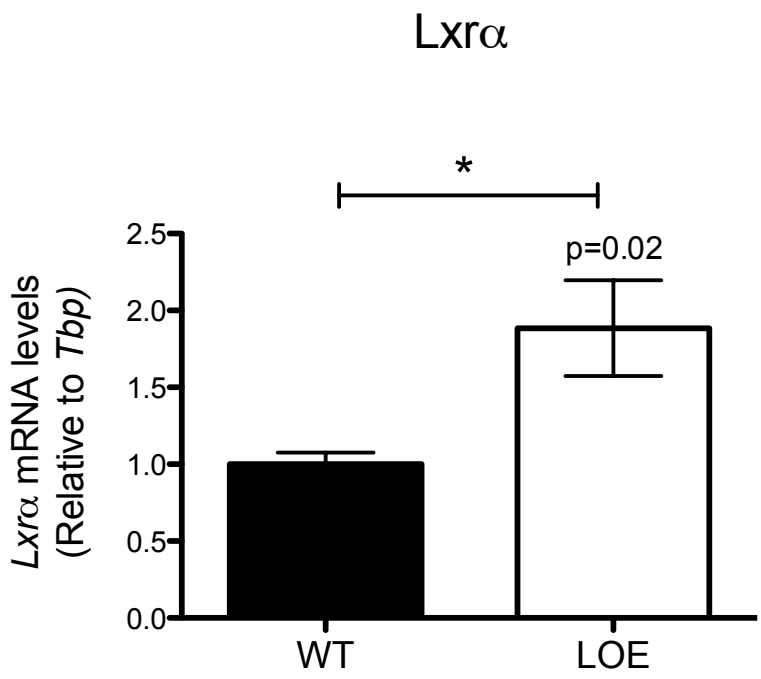


Figure 3.4: Levels of hepatic *Lxrα* mRNA are increased in LOE mice compared to their wild-type littermates.

Total RNA was extracted from liver of chow-fed, 6-8 week old, male liver 11β-HSD1 overexpressor (LOE) mice and their wild-type (WT) littermates. *Lxrα* mRNA levels were measured by qPCR and are expressed relative to *Tbp* mRNA levels, used as an internal control. Values are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=6/group; * $p<0.05$.

3.3.3. No difference in hepatic *Cyp7a1* mRNA levels between wild-type control and LOE mice

Cholesterol 7α-hydroxylase (encoded by *Cyp7a1*) catalyses the rate-limiting step in the classical pathway of bile acid synthesis and, in mice, is a direct target of LXRα (Zhu *et al*., 2012; see 1.5.5). LXRα deficient mice lose the ability to upregulate *Cyp7a1* gene expression in response to increased intracellular cholesterol levels, and, therefore, accumulate cholesterol esters in the liver, eventually leading to impaired hepatic function (Peet *et al*., 1998). Despite the presence of LXRβ in LXRα deficient mice, it cannot substitute for LXRα in the regulation of *Cyp7a1* expression (Lehmann *et al*., 1997; Peet *et al*., 1998). Consistent with this, LXRβ deficient mice retain normal hepatic cholesterol metabolism, highlighting the key role of LXRα in this process (Alberti *et al*., 2001).

To determine whether hepatic induction of *Lxrα* in LOE mice leads to increased degradation of cholesterol via bile acid synthesis, *Cyp7a1* mRNA levels were measured in liver samples from chow-fed, 6-8 week old LOE mice and their WT littermates.

There was no difference in the hepatic levels of *Cyp7a1* mRNA between genotypes (Figure 3.5), suggesting no difference in hepatic cholesterol catabolism through bile acid synthesis between control WT and transgenic LOE mice.

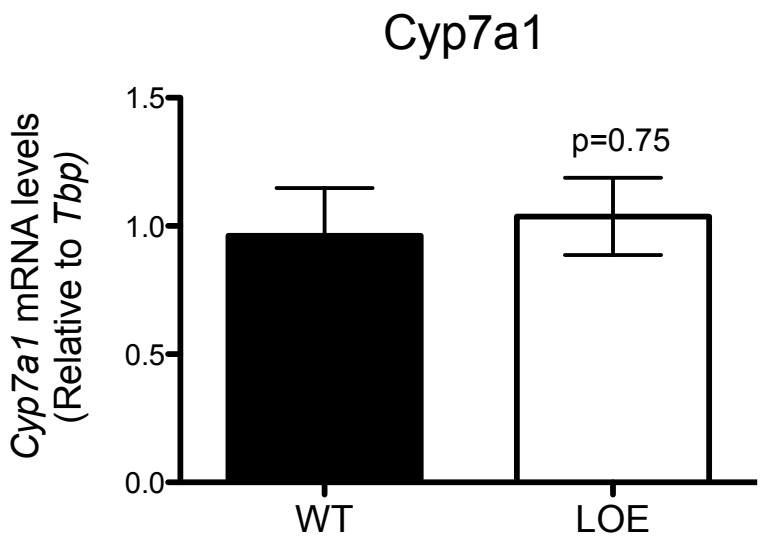


Figure 3.5: Similar hepatic *Cyp7a1* mRNA levels in WT control and LOE mice.

Total RNA was extracted from liver of chow-fed, 6-8 week old, male liver 11β-HSD1 overexpressor (LOE) mice and their wild-type (WT) littermates. *Cyp7a1* mRNA levels were measured by qPCR and are expressed relative to *Tbp* mRNA levels, used as an internal control. Values are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=6/group.

3.3.4. LOE mice have increased hepatic *Abcg8* mRNA levels

In addition to stimulation of bile acid synthesis, activation of LXR also induces hepatic cholesterol excretion by increasing transcription of the (hepatic) ATP-binding cassette (ABC) transporters G5 and G8 (Repa *et al*., 2002; Yu *et al*., 2003; Graf *et al.*, 2003; Hong *et al*., 2012; see 1.5.5). These transporters are expressed at the canalicular membrane of hepatocytes where they drive cholesterol transport into bile for excretion (Yu *et al*., 2002).

To determine whether LOE mice show activation of hepatic cholesterol secretion into bile, *Abcg5* and *Abcg8* mRNA levels were measured in liver from chow-fed, 6-8 week old LOE mice and their WT littermates.

LOE mice had increased hepatic *Abcg8* mRNA levels compared to WT control mice (Figure 3.6B). Although not reaching statistical significance, levels of *Abcg5* mRNA were increased to a similar extent in livers of LOE mice (Figure 3.6A). Together, these data suggest increased hepatic cholesterol secretion into bile in these mice.

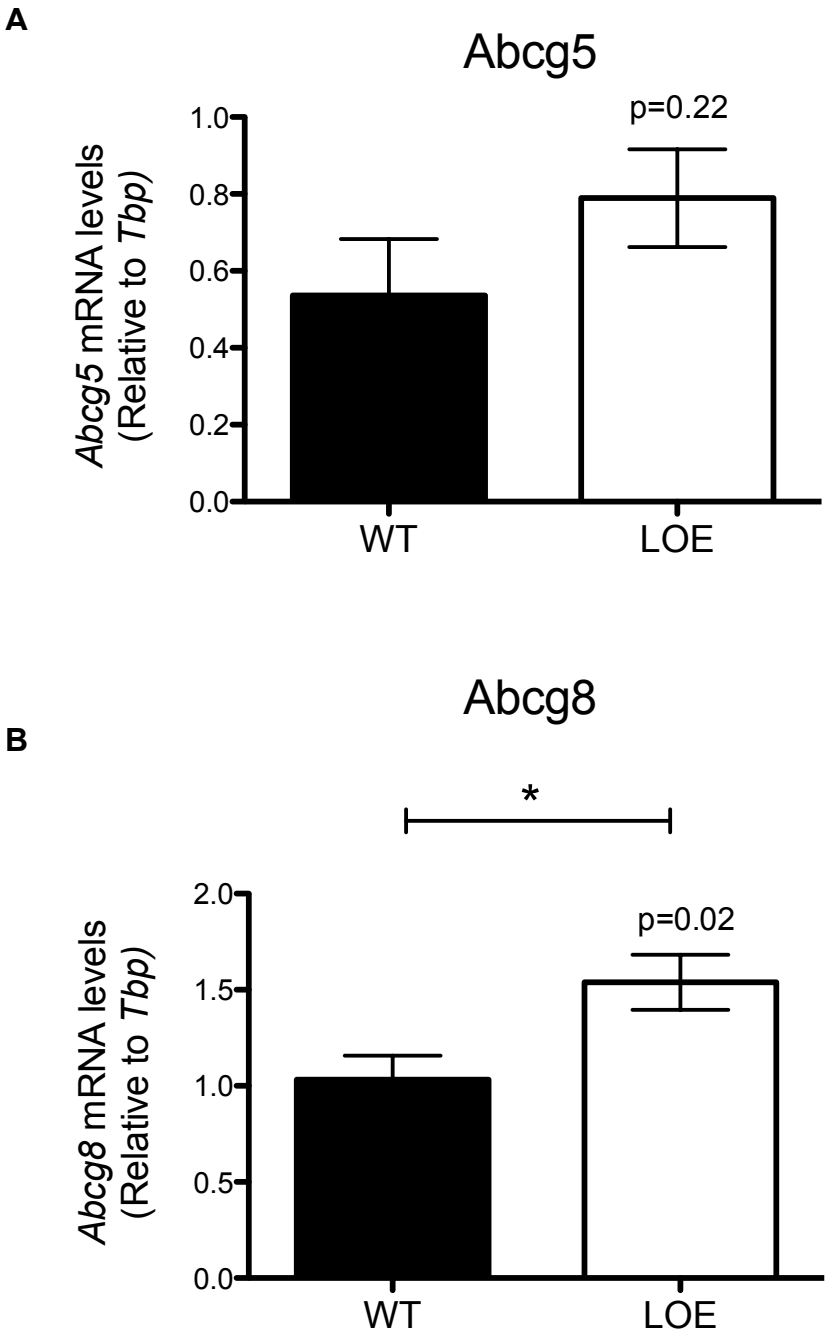


Figure 3.6: Levels of hepatic *Abcg8* mRNA are increased in LOE mice compared to WT controls.

Total RNA was extracted liver of chow-fed, 6-8 week old, male liver 11β-HSD1 overexpressor (LOE) mice and their wild-type (WT) littermates. Messenger RNA levels were measured by qPCR. **(A)** *Abcg5*, and **(B)** *Abcg8* mRNA levels are expressed relative to *Tbp* mRNA. Values are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=6/group; * $p<0.05$.

3.3.5. No change in hepatic *Abca1* mRNA levels between wild-type control and LOE mice

ABC transporter A1 (ABCA1) mediates cholesterol efflux from peripheral cells to lipid-poor apolipoproteins (apo-A1 and apoE), which then form HDL. In doing so, ABCA1 controls the rate-limiting step in HDL particle assembly, and is considered essential for RCT (Vaisman *et al*., 2001; Basso *et al*., 2003; Kang *et al*., 2013; see 1.5.4). Studies in mouse models have shown the crucial role of hepatic ABCA1 in the formation of nascent HDL particles; adenoviral and transgenic overexpression of hepatic ABCA1 raises plasma HDL concentrations (Vaisman *et al*., 2001; Basso *et al*., 2003; Wellington *et al*., 2003), whereas selective deletion of hepatic *Abca1* reduces plasma HDL levels by approximately 80% in chow-fed mice (Timmins *et al*., 2005). Numerous studies have also revealed that LXRα and LXRβ upregulate expression of rodent as well as human ABCA1 (in macrophages and intestine) (Repa *et al*., 2000b; Costet *et al*., 2000; Schwartz *et al*., 2000; Muscat *et al*., 2002; Sparrow *et al*., 2002; Kotokorpi *et al*., 2007; Mitro *et al*., 2007; Zhao *et al*., 2008).

To investigate whether increased expression of *Lxrα* in livers of LOE mice leads to upregulation of hepatic *Abca1* expression, *Abca1* mRNA levels were measured in liver samples of WT control and LOE mice.

There was no difference in the hepatic levels of *Abca1* mRNA between genotypes (Figure 3.7), suggesting no change in ABCA1-mediated HDL particle assembly between WT and LOE mice.

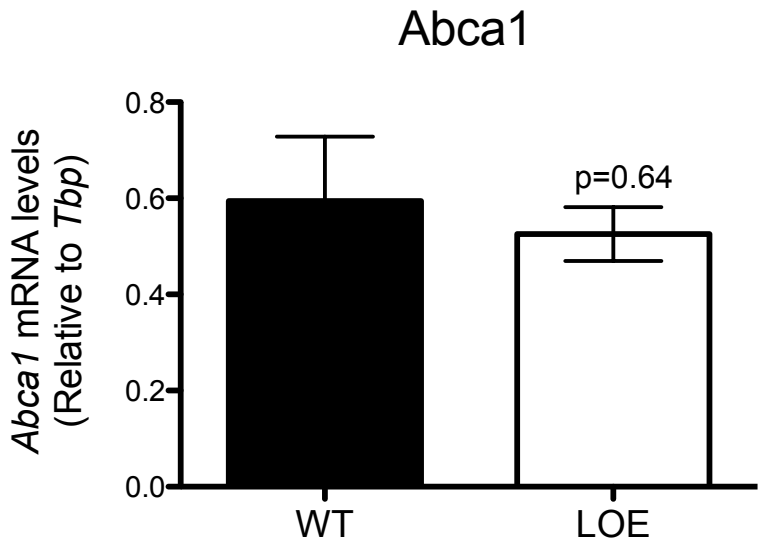


Figure 3.7: Similar hepatic *Abca1* mRNA levels in WT control and LOE mice.

Total RNA was extracted from liver of chow-fed, 6-8 week old, male liver 11β-HSD1 overexpressor (LOE) mice and their wild-type (WT) littermates. *Abca1* mRNA levels were measured by qPCR and are expressed relative to *Tbp* mRNA levels, used as an internal control. Values are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=6/group.

3.3.6. The initial finding of increased levels of cleaved SREBP-2 protein in LOE liver microsomes is not replicated in a new set of LOE liver microsomal samples

Preliminary data revealed increased cleaved SREBP-2 protein levels in liver microsomal fractions of LOE mice (Figure 3.2). Because cleaved/mature SREBP-2 protein activates transcription of target genes in nuclei of cells (Brown and Goldstein, 1997), western blot analysis was carried out to confirm that hepatic 11β-HSD1 overexpression leads to increased levels of cleaved SREBP-2 in *nuclear* fractions of liver from LOE and control mice. The liver nuclear as well as microsomal fractions used in Figure 3.8 were from new LOE and control liver samples (different to the samples used in Figure 3.2). 11β-HSD1 antibody was used as a marker for microsomes (11β-HSD1 is a microsomal protein (Ozols, 1995; Mziaut *et al*., 1999)) and showed the presence of 11β-HSD1 in nuclear fractions (Figure 3.8), suggesting microsomal contamination. Furthermore, cleaved SREBP-2 was clearly present to a similar extent in both LOE and wild-type microsomal fractions (Figure 3.8), which is inconsistent with the initial finding (Figure 3.2).

To examine if the initial samples still gave the same results as previously, two 'old' WT and two 'old' LOE liver microsomal fractions (used to generate Figure 3.2) were selected, at random, for western blot analysis. Despite weaker 124kDa bands (uncleaved SREBP-2), the initial findings were replicated here with respect to cleaved SREBP-2 protein levels; bands representing cleaved SREBP-2 protein were visible in LOE liver microsomes, but undetectable in WT liver microsomes (Figure 3.9).

In order to resolve the discrepancy in cleaved SREBP-2 between original and different, 'newer' samples, another cohort of WT control and LOE mice were used (separate from those in Figures 3.2 and 3.8), to obtain liver microsomal fractions for western blot analyses. These samples revealed the presence of cleaved SREBP-2 protein in liver microsomal fractions from LOE as well as control mice (Figure 3.10) with undetectable uncleaved SREBP-2 protein (Figure 3.10). These results are consistent with those in Figure 3.8, but still inconsistent with the original pilot study finding (Figure 3.2). Repeated experiments on different LOE and control liver samples gave results similar to those in Figures 3.8 and 3.10 (data not shown).

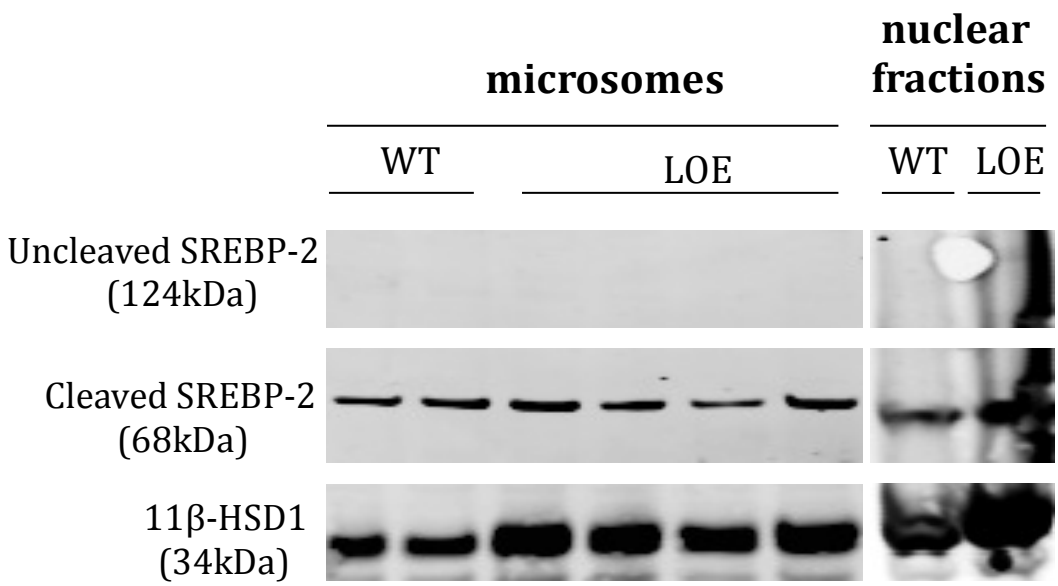


Figure 3.8: Cleaved SREBP-2 protein is present in both, WT and LOE liver microsomal fractions. Liver nuclear fractions reveal microsomal contamination.

Western blotting was used to examine SREBP-2 protein levels in liver microsomal fractions and liver nuclear fractions from wild-type control (WT) and liver 11β-HSD1 overexpressor (LOE) mice. Each lane contains 25μg protein from one mouse. The SREBP-2 antibody used detects uncleaved (124kDa) and cleaved (68kDa) SREBP-2 proteins. The 11β-HSD1 antibody was used as a marker for microsomes.

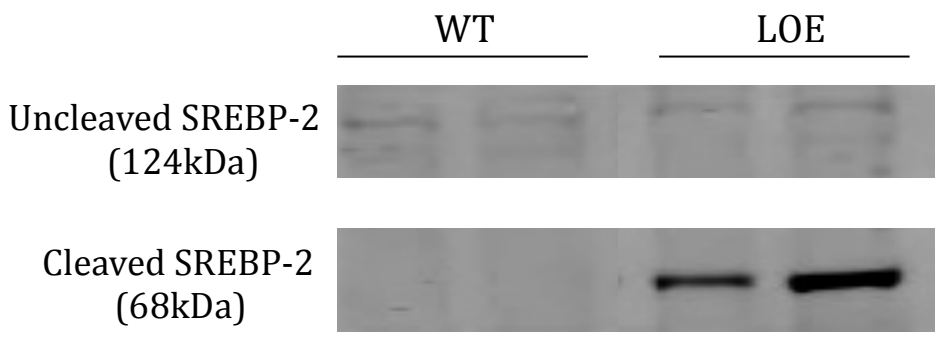


Figure 3.9: Initial findings (increased cleaved SREBP-2 protein in LOE liver microsomes) are replicated in the original WT and LOE liver microsomal fractions.

Western blotting was used to examine SREBP-2 protein levels in liver microsomal fractions from wild-type control (WT) and liver 11β-HSD1 overexpressor (LOE) mice. Each lane contains 25μg protein from one mouse, either WT or LOE as labelled appropriately. Here, some of the original liver microsomal fractions have been used. The SREBP-2 antibody used detects uncleaved (124kDa) and cleaved (68kDa) SREBP-2 proteins.

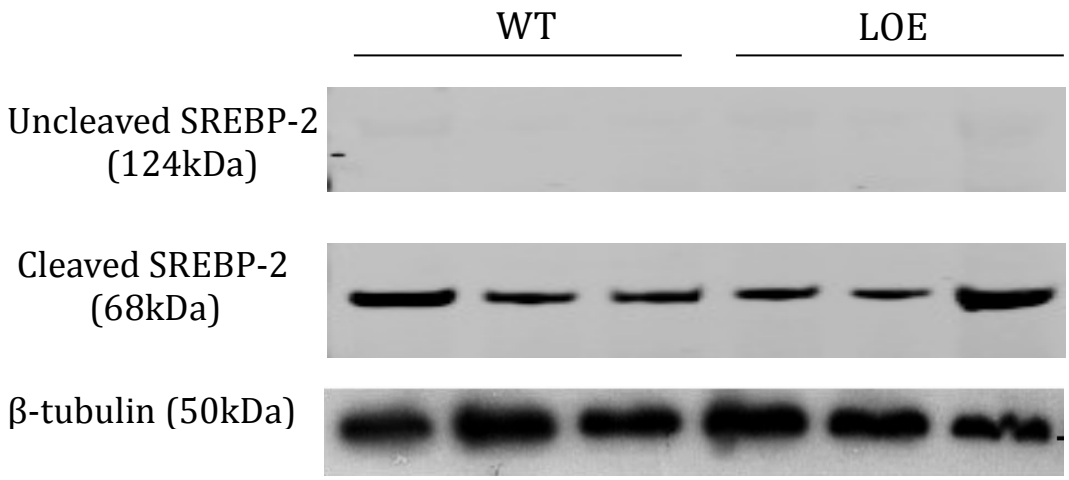


Figure 3.10: There is an inconsistency in cleaved SREBP-2 protein levels between liver microsomal fractions from original and new cohorts of WT control and LOE mice.

Western blotting was used to examine SREBP-2 protein levels in liver microsomal fractions from a new cohort of wild-type control (WT) and liver 11β-HSD1 overexpressor (LOE) mice. Each lane contains 30-35μg protein from one mouse, either WT or LOE as labelled appropriately. The SREBP-2 antibody used detects uncleaved (124kDa) and cleaved (68kDa) SREBP-2 proteins. The β-tubulin antibody was used as a loading control.

3.4. Discussion

These data show that, in contrast to the hypothesis, overexpression of hepatic 11β-HSD1 does *not* lead to increased expression of mRNAs encoding hepatic cholesterol biosynthetic enzymes. Increased cholesterol synthesis is accompanied by an increase in the activity and mRNA levels of HMG-CoA reductase (Istvan and Deisenhofer, 2001; Horton *et al*., 2002) as well as a coordinate increase in the mRNA levels of other key enzymes of the mevalonate pathway, such as HMG-CoA synthase, farnesyl diphosphate synthase, and squalene synthase (Harris *et al*., 1997). The mevalonate pathway is subject to negative end-product regulation (for example, by mevalonate, cholesterol, and other sterols such as oxysterols), and quantitatively, the most significant point of suppression is considered to be prior to mevalonate formation (Brown and Goldstein, 1980; Goldstein and Brown, 1990; Osborne, 1995). Among the enzymes that are essential to convert acetyl-CoA to mevalonate (the committed step in the synthesis of cholesterol), two enzymes have been clearly demonstrated to be under feedback regulation by cholesterol; microsomal HMG-CoA reductase (HMGCR) and cytoplasmic HMG-CoA synthase (HMGCS) (Clinkenbeard *et al*., 1975; Goldstein and Brown, 1990), therefore these were included in this study. Consistent with its role as the rate-limiting enzyme in cholesterol biosynthesis, HMGCR is also the target of statins, an efficacious therapeutic for the treatment of hyperlipidaemia and atherosclerotic cardiovascular disease, which act by inhibiting HMGCR in hepatocytes, thereby inhibiting cholesterol synthesis (Crouse *et al.,* 1995; Jukema *et al.,* 1995; Pitt *et al.,* 1995; Istvan and Deisenhofer, 2000; Istvan and Deisenhofer, 2001; Nissen, 2005; Nissen *et al.,* 2006). Glucocorticoids (GC) have been reported to inhibit cholesterol biosynthesis (Melnykovych *et al*., 1976; Russell, 1992; Russell, 2003) and Ramachandran *et al*. (1978) showed that GC-mediated inhibition of cholesterol biosynthesis in HeLa cells is due to HMGCS repression (Ramachandran *et al*., 1978). However, this remains controversial, for example dexamethasone and cortisol stimulated cholesterol synthesis up to 4-fold in human fetal hepatocytes (Carr and Simpson, 1983).

Paterson *et al*. (2004) previously observed a trend for increased hepatic *Hmgcr* mRNA expression in LOE mice, though this did not achieve significance due to high

inter-animal variability (Paterson *et al*., 2004). We thought this might be because the study was carried out on generation 3 backcross animals, where genetic variability could have contributed to phenotypic variability. Here, with mice congenic on the C57BL/6 background, there were still no differences in hepatic *Hmgcr* mRNA levels between LOE and control mice. This current study also measured hepatic *Hmgcs* mRNA levels in transgenic LOE and wild-type C57BL/6 mice, and like *Hmgcr*, hepatic *Hmgcs* mRNA levels did not differ between LOE and control mice. These results oppose the hypothesis of this chapter. At the molecular level, SREBP-2 governs cholesterol biosynthesis by upregulating the transcription of genes that encode enzymes of the cholesterol biosynthetic pathway (Horton *et al*., 1998; Horton *et al.*, 2002). Hence, it was surprising that an increase in the mature/cleaved form of SREBP-2 protein (preliminary finding) did not lead to increased hepatic expression of *Hmgcr* and *Hmgcs* mRNAs in LOE mice. However, it remained possible that an increase in *Hmgcr* and *Hmgcs* expression was not detected because of variation in the time that the tissues were collected in this study (between 13:00h and 14:00h), as diurnal variation occurs in both cholesterol synthesis and reductase expression with maximum activities observed during the dark period and minimum activities around noon (12:00h) (Edwards *et al*., 1972; Shefer *et al*., 1972; Jones and Schoeller, 1990; Cella, 1995; Jurevics, 2000).

Cholesterol is the precursor to oxysterol LXR ligands (Fu *et al*., 2001; Lund *et al*., 2003) and therefore, increased cholesterol synthesis is likely to lead to LXR activation (Forman *et al*., 1997; Janowski *et al*., 1999). This is consistent with the role of LXRα as a sterol "sensor", activated by the intracellular accumulation of cholesterol derivatives to stimulate cholesterol efflux, RCT and cholesterol excretion into bile (Joseph *et al*., 2002; Nomiyama and Bruemmer, 2008; Hong *et al*., 2012; Zhang *et al*., 2012). Therefore, although at first appearing to be contradictory to our hypothesis, it was predicted that liver overexpression of 11β-HSD1 leads to activation of hepatic *Lxrα* and its downstream targets involved in cholesterol efflux and excretion, in response to excess intracellular cholesterol due to increased cholesterol synthesis. There were, indeed, increased hepatic *Lxrα* mRNA levels in LOE mice compared to control mice. This is consistent with a previous study carried out by Paterson *et al.* (2004) which showed that LOE mice have increased hepatic *Lxrα* expression

compared to wild-type mice (Paterson *et al*., 2004). Several studies have demonstrated the existence of an autoregulatory loop controlling the expression of LXRα but not LXRβ (Laffitte *et al*., 2001; Whitney *et al*., 2001; Li *et al*., 2002). However, these studies show that autoregulatory upregulation of LXRα expression may be limited to human cells (Laffitte *et al.*, 2001; Whitney *et al*., 2001). Paterson *et al.* (2004) observed increased hepatic *Pparα* mRNA levels in LOE mice, and it is possible that PPARα induction, which is GC-sensitive (Lemberger *et al*., 1994), may upregulate LXRα expression in these mice (Chinetti *et al*., 2001; Chawla *et al*., 2001); for instance, either directly through a PPAR response element, or indirectly via increased levels of acetyl-CoA (produced by lipid oxidation) leading to cholesterol biosynthesis, and hence formation of oxysterol LXR ligands.

Cholesterol conversion into bile acids in the liver is a pivotal pathway to reduce serum cholesterol levels. Bile acid synthesis and excretion represent the major pathway of cholesterol removal from the body (Russell, 2003). Cholesterol 7-alpha-hydroxylase (CYP7A1) is the rate-limiting enzyme in the classical bile acid biosynthetic pathway, accounting for at least 75% of the total bile acid pool (Chiang, 2004). It has been shown that in mice and rats, but not humans, LXRα activation induces the expression of *Cyp7a1*, thus promoting cholesterol degradation (Janowski *et al*., 1996; Lehmann *et al*., 1997; Peet *et al*., 1998; Chiang *et al*., 2001). Furthermore, *Cyp7a1* expression is GC-inducible (Princen *et al*., 1989). However, surprisingly, no difference was observed in hepatic *Cyp7a1* mRNA levels between LOE and control mice, suggesting that there is no change in cholesterol removal via bile acid synthesis between genotypes. This is not consistent with the study carried out by Paterson *et al*. (2004), which reported increased hepatic *Cyp7a1* expression in LOE mice (Paterson *et al*., 2004). In this previous study, Northern blotting was used to investigate hepatic gene expression, and although there was no difference in the 4.5kb *Cyp7a1* transcript, the authors observed an increase in a 7.1kb *Cyp7a1* transcript in LOE mice, and reported an overall increase in hepatic *Cyp7a1* mRNA in these mice (Paterson *et al*., 2004). However, database searching using the BLAST bioinformatics program and examination of Ensembl genome browser for the mouse *Cyp7a1* gene only revealed a 4.5kb transcript for *Cyp7a1* (not shown here), without

an alternative 7.1kb transcript. Therefore, the identity of the 7.1kb transcript in the previous study remains unclear.

LOE mice had elevated levels of hepatic *Abcg5* (though not reaching statistical significance) and *Abcg8* mRNA. This is consistent with both *Abcg5* and *Abcg8* being direct targets of LXR activation (Berge *et al*., 2000; Lee *et al*., 2001; Repa *et al*., 2002). These data suggest increased hepatobiliary cholesterol secretion in LOE mice as the induction of hepatic ABCG5 and ABCG8 has been demonstrated to promote cholesterol excretion into bile (Graf *et al*., 2002; Yu *et al*., 2002; Yamazaki *et al*., 2011). This also possibly accounts for normal plasma and liver cholesterol levels observed in (control diet-fed) LOE mice (Paterson *et al*., 2004). However, LOE mice also exhibit fatty liver, which was confirmed to be the result of elevated hepatic TG levels and this was attributed to LXRα-regulated FAS induction (Joseph *et al*., 2002b) in these mice (Paterson *et al*., 2004).

The removal of cholesterol through bile acid production occurs exclusively in the liver, therefore, excess cholesterol from other tissues must be returned to the liver for catabolic elimination. As previously mentioned, RCT is the process by which HDL particles mediate the transport of cholesterol from peripheral tissues to the liver. Studies in mouse models have shown the crucial role of *hepatic* ABCA1 in HDL biogenesis (Vaisman *et al*., 2001; Basso *et al*., 2003; Wellington *et al*., 2003; Timmins *et al*., 2005). It has also been shown that LXR activation upregulates expression of ABCA1 in macrophages (Repa *et al*., 2000b; Costet *et al*., 2000; Schwartz *et al*., 2000; Muscat *et al*., 2002; Mitro *et al*., 2007; Zhao *et al*., 2008). However, no differences were observed in hepatic *Abca1* mRNA levels between LOE and WT mice. This finding does not rule out the possibility of active ABCA1-mediated cholesterol efflux in LOE macrophages and/or cholesterol efflux mediated by ABCG1 and it will be of interest to measure this in the future.

Western blot analyses to examine cleaved SREBP-2 protein levels in liver nuclear fractions of LOE mice were not successful due to microsomal contamination. Furthermore, despite cleaner blots and extensive attempts, the original finding (of increased cleaved SREBP-2 protein in LOE liver microsomes) was not repeated in

separate cohorts of LOE and control liver microsomal samples. Hence, the preliminary data on the effect of liver 11β-HSD1 overexpression upon levels of cleaved SREBP-2 protein are not conclusive. The reason behind this remains unclear.

In summary, there is no evidence that liver 11β-HSD1 overexpression promotes cholesterol synthesis by increasing levels of mRNAs encoding hepatic cholesterol biosynthetic enzymes, at least in chow-fed LOE mice. However, unchanged gene expression does not exclude posttranscriptional/posttranslational regulation of enzyme activities. As predicted, overexpression of liver 11β-HSD1 leads to increased expression and activity of *Lxrα*, possibly in response to increased intracellular cholesterol levels. Despite similarities in some results with those in the initial study carried out by Paterson *et al*. (2004), the current data display some differences. There is no evidence in the present study for increased bile acid synthesis in LOE mice. However, elevated hepatic *Abcg5/8* mRNA expression in LOE mice suggests a role for increased hepatic 11β-HSD1 in secretion of cholesterol into the biliary lumen, possibly mediated through higher *Lxrα* expression in these mice.

Chapter 4. Effect of 11β-HSD1 on Cholesterol Homeostasis with Fat and Cholesterol Feeding

4.1. Introduction

Lipid metabolism and homeostasis are crucially dependent on diet. Although dietary fatty acids play an important role in cellular physiology by altering membrane fluidity, signal transduction and gene expression (Duplus *et al*., 2000; Dussault and Forman, 2000; Madsen *et al*., 2005), in excess, they are implicated in the development of metabolic syndrome and cardiovascular disease. Trans fatty acids (TFA) and saturated fatty acids (SFA) are associated with the development of obesity and insulin resistance (Lefebvre *et al*., 1999; Lovejoy *et al*., 2001; Dorfman *et al*., 2009). Fatty diets lead to high levels of plasma TG, which have also been linked to increased risk of atherosclerosis, heart disease and stroke (Labreuche *et al*., 2009). However, this is debatable, as recent reports show no significant association between intake of saturated fats and cardiovascular disease (Siri-Tarino *et al*., 2010; Chowdury *et al*., 2014). Since Western-style diets deliver fat mainly from animal sources, they are also cholesterol-rich. In humans, consumption of a Western-style diet has been reported to elevate plasma/serum total cholesterol and LDL levels, and increase coronary heart disease mortality (Sacks *et al*., 1984; Ginsberg *et al*., 1994; Riemersma, 2002; Eilat-Adar *et al.*, 2013). Feeding cholesterol-rich diets to wild-type C57BL/6 mice shows a switch in lipoprotein profile from HDL to LDL (Morton *et al*., 2004), and in atherosclerosis-prone mouse models (*Apoe*$^{-/-}$ and LDLR$^{-/-}$ mice), high cholesterol diets lead to increased total cholesterol and LDL levels as well as extensive atherosclerosis (Ishibashi *et al*., 1994; van Ree *et al*., 1994; Zhang *et al*., 1994; Meir and Leitersdorf, 2004).

Although no differences were observed in hepatic *Hmgcr* and *Hmgcs* mRNA levels between chow-fed LOE mice and WT controls (Chapter 3), it is likely that diet is an influential factor. 7-Ketocholesterol is formed non-enzymatically by free radical oxidation of cholesterol (Hodis *et al*., 1991), or from dietary intake of cholesterol-rich food (Nielsen *et al*., 1995; Vine *et al*., 2002). Based on the original hypothesis that hepatic 11β-HSD1 converts 7-KC to 7β-HC and, therefore, removes an endogenous brake on cholesterol synthesis in liver, feeding LOE mice a cholesterol-rich diet is predicted to increase 7-KC availability for liver 11β-HSD1, hence driving synthesis of 7β-HC, and thus increasing hepatic cholesterol biosynthesis in cholesterol-fed LOE

mice. Moreover, chow-fed LOE mice showed evidence for increased biliary cholesterol excretion compared to WT controls (Chapter 3) and it would be interesting to observe the impact of dietary risk factors.

To investigate the *in vivo* effects of 11β-HSD1 and dietary risk factors upon hepatic cholesterol homeostasis, a dietary experiment was carried out in liver 11β-HSD1 overexpressor (LOE), wild-type (WT) C57BL/6, and 11β-HSD1 deficient (HSD1 KO) mice. Liver-specific 11β-HSD1 knockout mice were not used in this experiment as their generation was underway at the time. Male WT, LOE and HSD1 KO mice (5-6 week old, n=6-10/group) were fed standard chow (C; 11% fat), high fat (HF; 58% fat) or western diet (WD; 41% fat + 0.21% cholesterol) for 12 weeks. Cholesterol-rich WD was used for the above-mentioned reasons, and HF diet was included to investigate effects of 11β-HSD1 on cholesterol homeostasis when precursor for cholesterol synthesis (acetyl CoA) is increased. Also, these particular diets have been used in previous studies within the lab (Paterson *et al*., 2004; Morton *et al*., 2004; Kipari *et al*. 2013). Although a matched low-fat diet would be more suitable as a control for the HF diet utilised in this study, a standard chow was used, which served as a control diet for both WD and HF diet. Apart from the effects on cholesterol homeostasis, other metabolic parameters such as weight gain were being investigated in this study (see 4.2). Although an increase in body weight can be noticed in 2-3 weeks of HF-feeding, the increase is gradual (Wang and Liao, 2012) and, therefore, the mice in this study were fed these diets for 12 weeks (long enough for significant weight gain). Body weight was measured weekly. Liver and fat depots were collected when the mice were culled at the end of the experiment.

4.2. Hypothesis and Aims

Hepatic overexpression of 11β-HSD1 will increase 7-KC to 7β-HC conversion in liver, removing the brake on cholesterol biosynthesis in this tissue. With a supply of 7-KC substrate through cholesterol-rich WD, for the above-mentioned reaction, higher levels of hepatic cholesterol synthesis are predicted on WD. On the other hand, 11β-HSD1 deficiency will result in greater levels of 7-KC on WD, providing an endogenous brake on cholesterol biosynthesis. Therefore, 11β-HSD1 deficiency is predicted to reduce hepatic cholesterol synthesis in mice fed WD. On HF diet, levels of acetyl CoA precursor will be greater, and so increased cholesterol biosynthesis is predicted in LOE mice. Conversely, HSD1 KO mice will be protected from this adverse metabolic phenotype.

Hypothesis: With high fat/cholesterol feeding, increased liver 11β-HSD1 will promote hepatic cholesterol biosynthesis via hepatic induction of SREBP-2 target genes in the cholesterol biosynthetic pathway. The opposite effect will be observed with knockout of 11β-HSD1.

Aims

1) To investigate metabolic parameters such as body, liver and adipose tissue weights in mice with 11β-HSD1 manipulations fed either standard chow, HF or cholesterol-rich diet.

2) To determine if mRNAs encoding cholesterol biosynthetic enzymes (SREBP-2 targets) are altered in livers of mice with 11β-HSD1 manipulations faced with HF or cholesterol challenge.

3) To test if mRNAs encoding LXRα and its downstream targets involved in cholesterol efflux and cholesterol clearance are altered in livers of mice with 11β-HSD1 manipulations challenged with HF or cholesterol diets.

4.3. Results

4.3.1. Significant effects of genotype and diet on body weight gain

Previous data revealed reduced body weight gain in HSD1 KO mice fed a HF diet compared to wild-type controls on the same diet (Morton *et al*., 2004). Paterson *et al*. (2004) reported no differences in body weight between HF-fed LOE and control mice but the effect of cholesterol-rich diet was not tested in their study.

Effects of HF and cholesterol-rich WD feeding on body weight gain were assessed in WT, LOE and HSD1 KO mice in this study.

After 12 weeks of chow, HF diet or WD, significant effects of diet and genotype were observed on body weight gain, with a significant interaction between genotype and diet (Figure 4.1). As expected, WT and LOE mice gained more weight with HF- and WD-feeding compared to chow diet (Figure 4.1). However, the same was not observed in HSD1 KO mice, which showed greater body weight gain on chow diet compared to WT mice on the same diet (Figure 4.1).

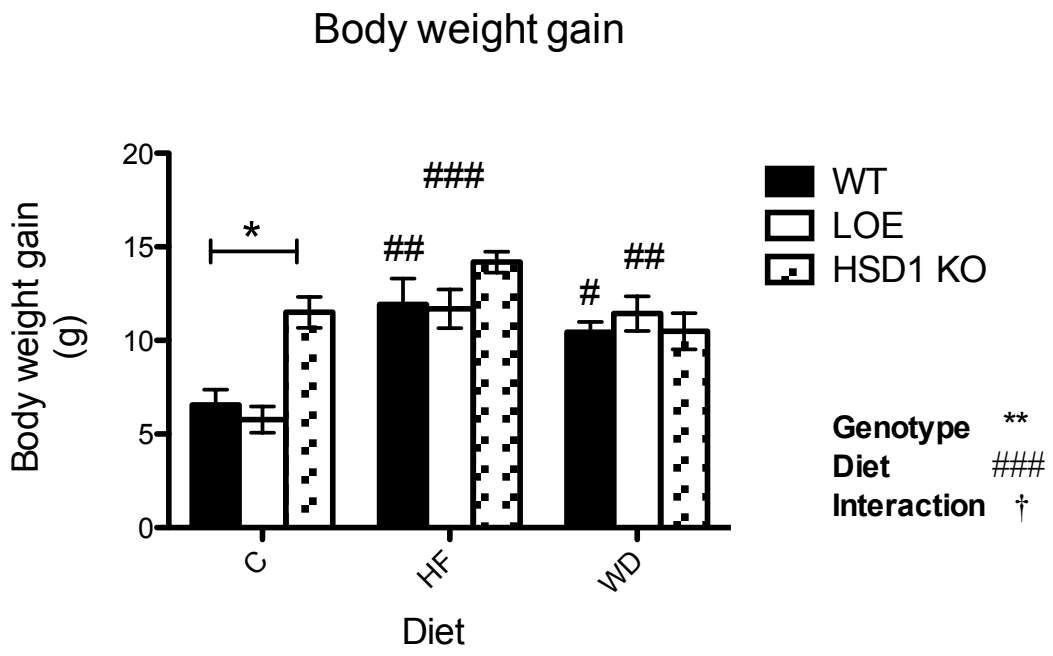


Figure 4.1: Significant effects of genotype and diet are observed on body weight gain.

Male, 5-6 week old wild-type (WT) C57BL/6, liver 11β-HSD1 overexpressor (LOE) and 11β-HSD1 deficient (HSD1 KO) mice were fed Chow (C), High Fat (HF) or Western (WD) diet for 12 weeks, and had their body weight gain measured. Values are means ± SEM and were analysed by two-way ANOVA with Tukey's multiple comparisons *post-hoc* test; n=8-10/group; * $p<0.05$, ** $p<0.01$ for effect of genotype; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ for effect of diet; † $p<0.05$ for interaction between genotype and diet.

4.3.2. Significant effects of genotype and diet on liver weight

The liver plays a major role in regulating metabolic homeostasis and is vital for nutrient metabolism. Previous work has shown that increased levels of dietary fat intake are associated with increased lipid accumulation in the liver, leading to fatty liver disease (Deng *et al*., 2005; Zou *et al*., 2006; Sathiaraj *et al*., 2011). Also, Paterson *et al*. (2004) observed a fatty liver phenotype in LOE mice on both control and HF diet (Paterson *et al*., 2004).

Following chow, HF or WD feeding, liver weights of WT, LOE and HSD1 KO mice (which were 17-18 weeks of age at the end of the experiment) were measured.

Although two-way ANOVA analysis showed significant effects of genotype and diet on liver weight (Figure 4.2), the Tukey's *post-hoc* test did not detect any specific genotype or diet differences. This might be due to a combination of the design of the experiment (2 factors, but 3 levels each) and the stringency of the *post-hoc* test. A Fisher's LSD test (which does not correct for multiple comparisons) revealed significantly reduced liver weight in WD-fed HSD1 KO mice compared to WD-fed WT mice (Figure 4.2). As for diet differences, decreased liver weights were observed in all genotypes with HF feeding, and in HSD1 KO mice with WD feeding (Figure 4.2).

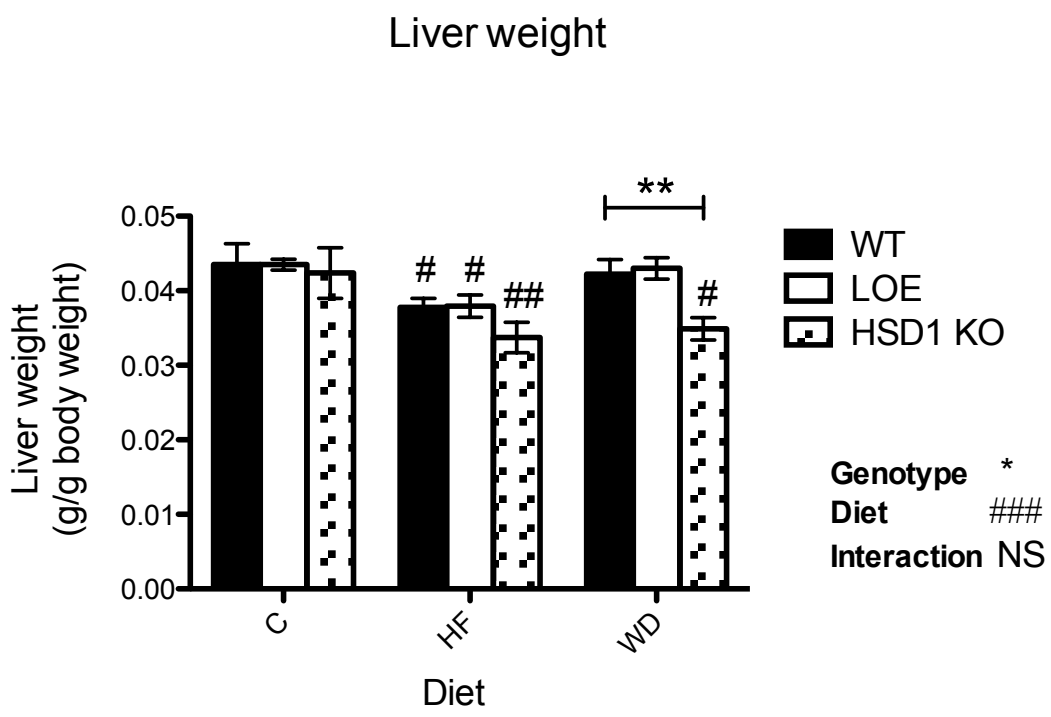


Figure 4.2: Significant effects of genotype and diet are observed on liver weights.

Male, 5-6 week old wild-type (WT) C57BL/6, liver 11β-HSD1 overexpressor (LOE) and 11β-HSD1 deficient (HSD1 KO) mice were subject to 12-week Chow (C), High Fat (HF) or Western (WD) diet, and had their terminal liver weights measured and normalised to body weights. Data are means ± SEM and were analysed by two-way ANOVA with Fisher's LSD *post-hoc* test; n=8-10/group; * $p<0.05$, ** $p<0.01$ for effect of genotype; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ for effect of diet; NS, not significant.

4.3.3. Significant effect of diet on white adipose tissue weight

Diet-induced obesity (via HF diet and/or high-caloric food) is characterised by an increase in adipose tissue, resulting mainly from the excessive storage of TG within adipocytes. The literature points out that the adverse metabolic consequences of obesity are predicted by the quantity of visceral fat, not just the total adipose mass (Vague, 1956; Montague and O'Rahilly, 2000). Dyslipidaemia, fatty liver disease and diabetes are closely associated with abdominal obesity (increased visceral fat), which is also a feature of metabolic syndrome (Marchesini *et al*., 2003).

To evaluate the distribution of adiposity in WT, LOE and HSD1 KO mice in this study, the weight of three different fat depots was measured: subcutaneous fat, epididymal fat and mesenteric fat.

In all genotypes, WT, LOE as well as HSD1 KO mice, WD feeding increased subcutaneous (Figure 4.3), epididymal (Figure 4.4) and mesenteric (Figure 4.5) fat weights, compared to standard chow diet. Similarly, HF feeding increased weights of all white fat depots in WT and HSD1 KO mice (Figures 4.3, 4.4 and 4.5). Two-way ANOVA showed no significant effect of genotype on subcutaneous (Figure 4.3) and epididymal (Figure 4.4) fat weights, but with respect to mesenteric fat weight, a significant effect of genotype was observed and HF-fed LOE mice displayed decreased mesenteric fat weight compared to HF-fed WT mice (Figure 4.5).

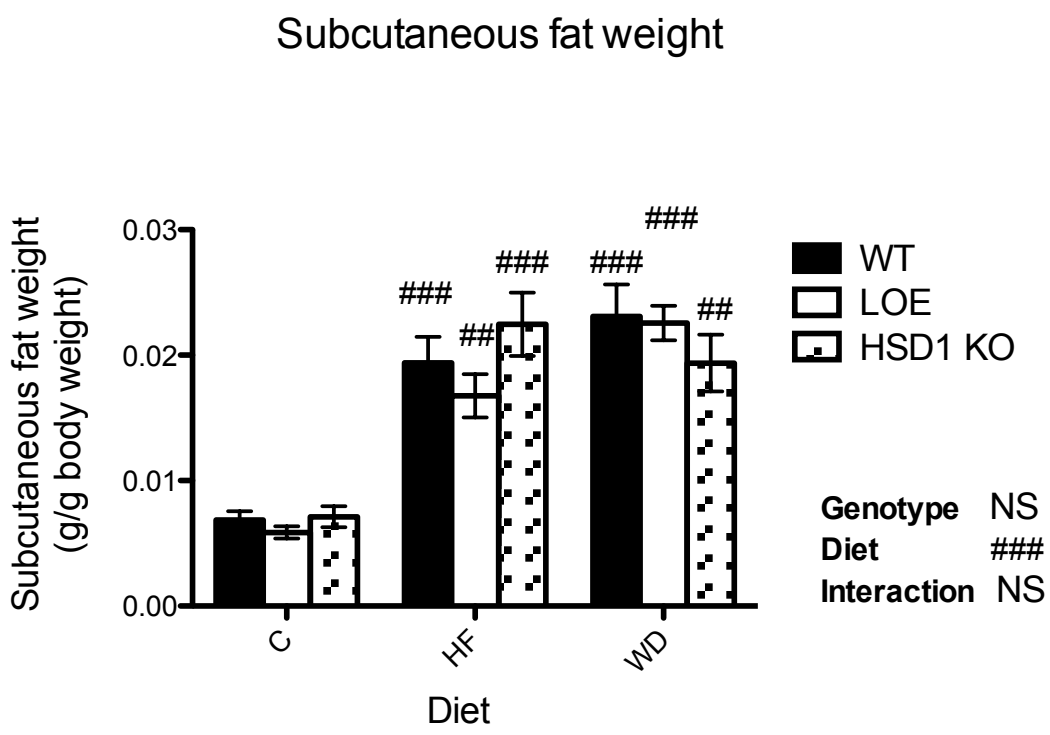


Figure 4.3: Significant effect of diet is observed on subcutaneous fat weights.

Male, 5-6 week old wild-type (WT), liver 11β-HSD1 overexpressor (LOE) and 11β-HSD1 deficient (HSD1 KO) mice were fed Chow (C), High Fat (HF) or Western (WD) diet for 12 weeks, and had their terminal subcutaneous fat weights measured and normalised to body weights. Values are means ± SEM and were analysed by two-way ANOVA with Tukey's multiple comparisons *post-hoc* test; n=8-10/group; ## $p<0.01$, ### $p<0.001$ for effect of diet; NS, not significant.

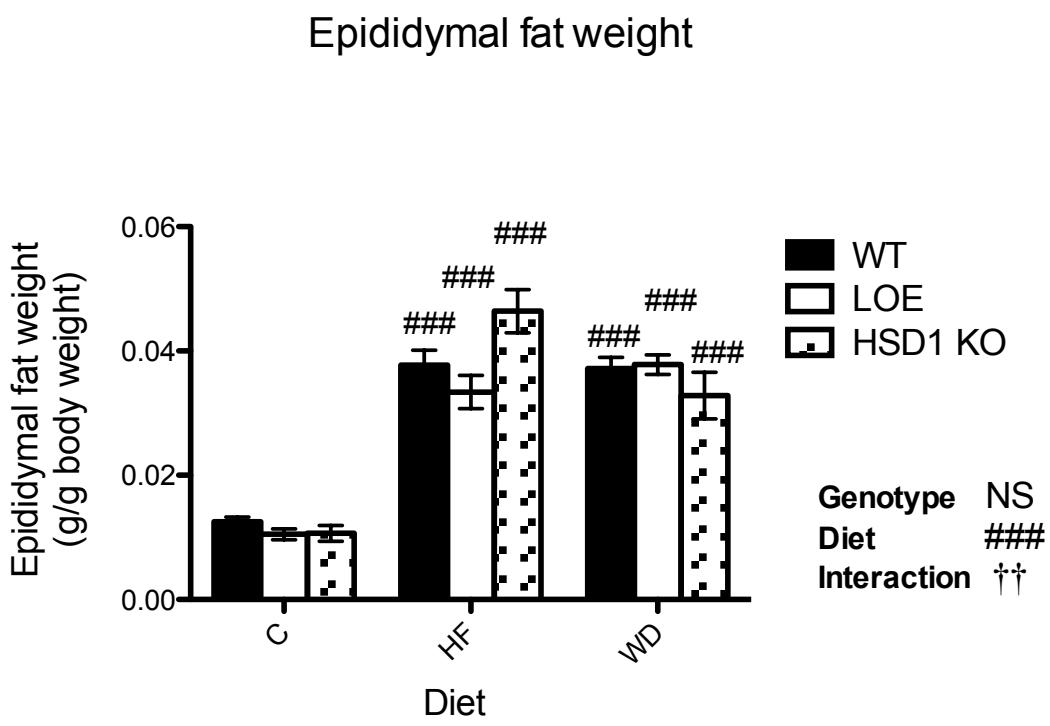


Figure 4.4: Significant effect of diet is observed on epididymal fat weights.

Epididymal fat weights of 5-6 week old male wild-type (WT) C57BL/6, liver 11β-HSD1 overexpressor (LOE) and 11β-HSD1 deficient (HSD1 KO) mice, which were fed Chow (C), High Fat (HF) or Western (WD) diet for 12 weeks, were normalised to body weights. Values are means ± SEM and were analysed by two-way ANOVA with Tukey's multiple comparisons *post-hoc* test; n=8-10/group; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ for effect of diet; †† $p<0.01$ for interaction between genotype and diet; NS, not significant.

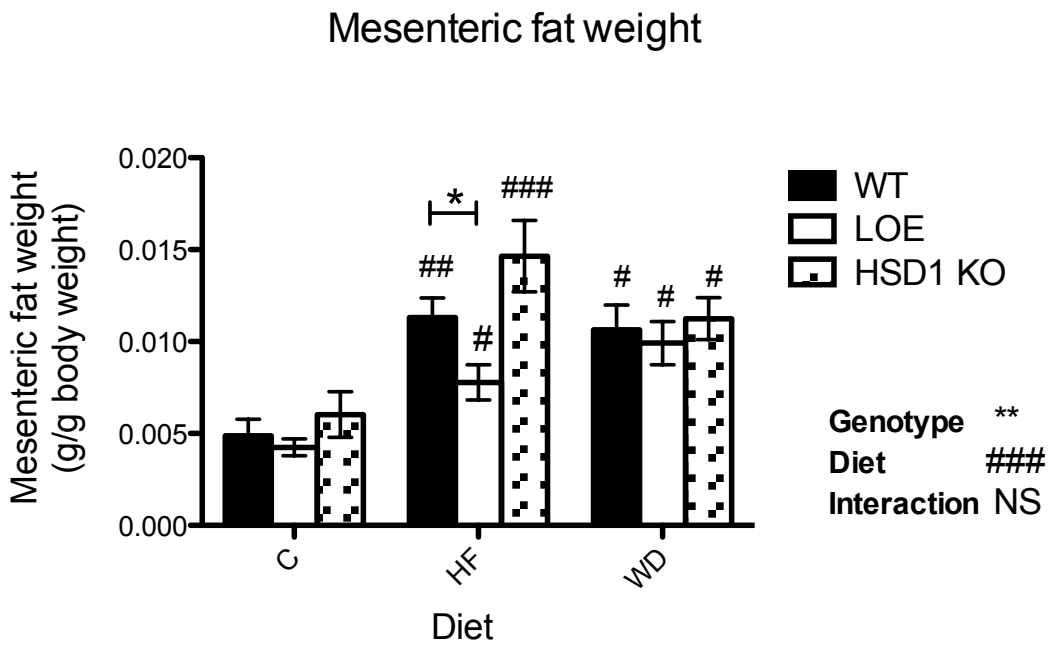


Figure 4.5: Significant effects of genotype and diet are observed on mesenteric fat weights.

Male, 5-6 week old wild-type (WT), liver 11β-HSD1 overexpressor (LOE), and 11β-HSD1 deficient (HSD1 KO) mice that were fed Chow (C), High Fat (HF) or Western (WD) diet for 12 weeks had their terminal subcutaneous fat weights measured and normalised to body weights. Values are means ± SEM and were analysed by two-way ANOVA with Tukey's multiple comparisons *post-hoc* test; n=8-10/group; * $p<0.05$, ** $p<0.01$ for effect of genotype; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ for effect of diet; NS, not significant.

4.3.4. Significant effect of diet on hepatic *Srebp2* mRNA levels

High-cholesterol diets suppress cholesterol synthesis in the livers of experimental animals. As the key regulator of cholesterol biosynthesis, SREBP-2 is expected to be significantly reduced with cholesterol feeding. This, indeed, has been observed in numerous studies (Hua *et al*., 1996; Shimomura *et al*., 1997; Vallim and Salter, 2010; Boone *et al*., 2011). Cholesterol feeding leads to intracellular cholesterol accumulation thereby inhibiting the cleavage/maturation of SREBP-2 (Brown and Goldstein, 1997; Goldstein *et al*., 2002; Vallim and Salter, 2010). Moreover, the *SREBP2* gene contains an active sterol response element (SRE) and has been shown to negatively autoregulate its own transcription (Sato *et al*., 1996), leading to a further decrease in mature/nuclear SREBP-2. Fat feeding has either little effect on SREBP-2 expression (Xu *et al*., 1999; Kim *et al*., 1999; Mater *et al*., 1999; Yahagi *et al*., 1999; Buettner *et al*., 2006), or, like cholesterol-rich diets, reduces SREBP-2 levels (Worgall *et al*., 1998; Thewke *et al*., 1998; Vallim and Salter, 2010; Shin *et al*., 2013). Paterson *et al*. (2004) showed no alteration in hepatic *Srebp2* expression in HF-fed LOE mice compared with control low fat-fed WT littermates. The effect of cholesterol feeding on LOE mice has not been investigated. As for *Srebp2* expression with 11β-HSD1 deficiency, a recent study that used an antisense oligonucleotide to knockdown 11β-HSD1 in livers of C57BL/6 mice showed no change in hepatic *Srebp2* mRNA levels with 11β-HSD1 knockdown (Li *et al*., 2011).

It was hypothesised that with high fat/cholesterol feeding, increased liver 11β-HSD1 will promote hepatic cholesterol biosynthesis via induction of SREBP-2 target genes involved in the cholesterol biosynthetic pathway (see 4.2). To examine *Srebp2* expression in livers of HF- and WD-fed LOE and HSD1 KO mice, hepatic *Srebp2* mRNA levels were measured via qPCR. WT C57BL/6 mice served as controls. For gene expression analysis in this study, six samples were chosen per group (n=6/group).

A significant effect of diet was observed on hepatic *Srebp2* mRNA levels. As expected, WT mice showed reduced hepatic *Srebp2* mRNA levels on HF diet and WD compared to chow diet (Figure 4.6). LOE and HSD1 KO mice also exhibited decreased hepatic *Srebp2* mRNA levels with WD feeding (Figure 4.6). The two-way

ANOVA showed a significant interaction between genotype and diet (Figure 4.6). Chow-fed HSD1 KO mice showed decreased hepatic levels of *Srebp2* mRNA compared to WT mice on the same diet (Figure 4.6). Surprisingly, there were also reduced hepatic *Srebp2* mRNA levels in chow-fed LOE mice compared to chow-fed WT mice (Figure 4.6).

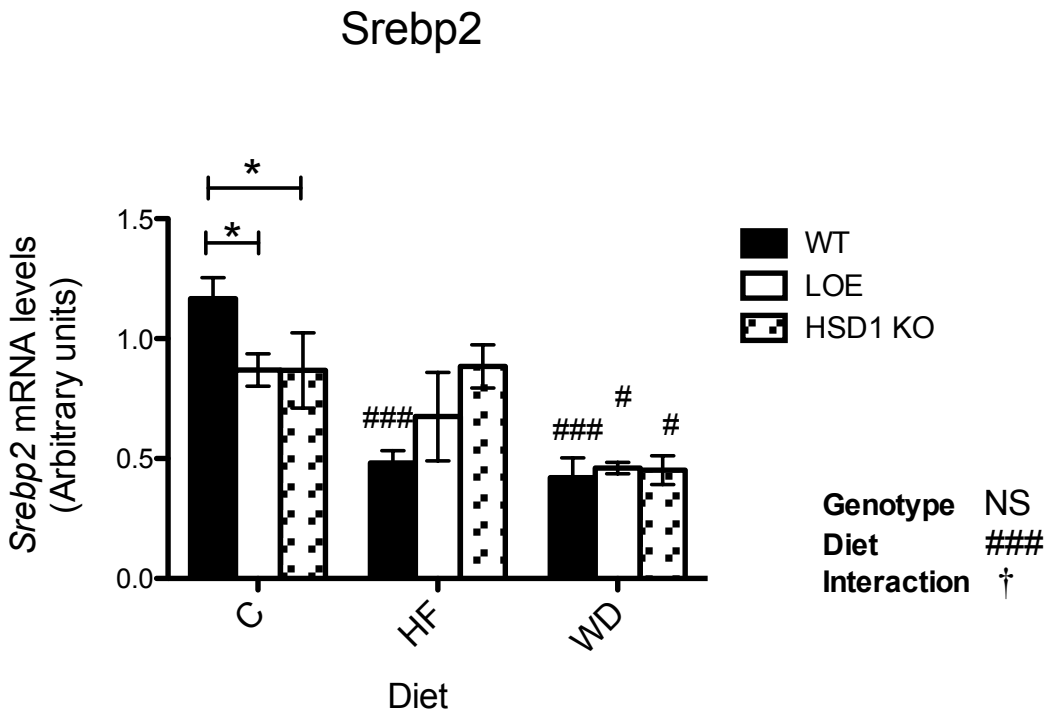


Figure 4.6: Significant effect of diet is observed on hepatic *Srebp2* mRNA levels.

Total RNA was extracted from livers of male wild-type control (WT), liver 11β-HSD1 overexpressor (LOE) and 11β-HSD1 knockout (HSD1 KO) mice fed one of the following diets for 12 weeks from 5-6 weeks of age; chow (C), high fat (HF) or western diet (WD). Hepatic *Srebp2* mRNA levels were measured by qPCR. Levels of *Srebp2* mRNA are expressed relative to the average of *Hprt* and *Actb* mRNAs, used as internal controls. Values are means ± SEM and were analysed by two-way ANOVA with Fisher's LSD *post-hoc* test; n=6/group; * $p<0.05$ for effect of genotype; # $p<0.05$, ### $p<0.001$ for effect of diet; † $p<0.05$ for interaction between genotype and diet; NS, not significant.

4.3.5. Reduced hepatic *Hmgcr* and *Hmgcs* mRNA levels in WT and LOE mice with WD feeding

It has been recognised for a long time that feeding cholesterol to animals decreases the rate of hepatic cholesterol synthesis to maintain intracellular cholesterol homeostasis (Gould and Taylor, 1950; Gould, 1951; Tomkin *et al*., 1953; Langdon and Bloch, 1953), and numerous studies have shown that this is primarily due to decreased activity and reduced mRNA levels of enzymes involved in the cholesterol biosynthetic pathway particularly HMGCR and HMGCS (Limi, 1967; Kandutsch and Packie, 1970; White and Rudney, 1970; Shapiro and Rodwell, 1971; Ness *et al*., 1991; Rudling, 1992; Lin *et al*., 1992; Kushwaha *et al*., 1995; Maxwell *et al*., 2003; Mutungi *et al*., 2007; Boone *et al*., 2011; see 1.4.1.1). Some studies have shown that HF feeding in C57BL/6 mice also reduces *Hmgcr* mRNA levels (Rudling, 1992), and HMGCR activity (Sumiyoshi *et al*., 2006). However, other studies demonstrate that HF feeding elevates *Hmgcr* mRNA (Lin *et al*., 2005; Wu *et al*., 2013) as well as HMGCR protein and enzyme activity (Wu *et al*., 2013). Paterson *et al*. (2004) demonstrated a trend for increased hepatic *Hmgcr* mRNA levels in control diet-fed LOE mice, which was abolished with HF feeding. The effect of cholesterol feeding on expression of genes encoding cholesterol biosynthetic enzymes in LOE mice has not been investigated.

To examine if cholesterol synthesis was altered in HF- and WD-fed LOE and HSD1 KO mice, via altered expression of SREBP-2 target genes in the cholesterol biosynthetic pathway, *Hmgcr* and *Hmgcs* mRNA levels were measured in livers from male 17-18 week old WT, LOE, and HSD1 KO mice following dietary intervention as described above.

A significant effect of diet was observed on hepatic *Hmgcr* and *Hmgcs* mRNA levels (Figure 4.7A and Figure 4.7B). While WT mice displayed a decrease in hepatic *Hmgcr* mRNA with both HF and WD feeding (Figure 4.7A), hepatic *Hmgcs* mRNA levels were significantly reduced only in WD-fed WT mice compared to chow-fed WT mice (Figure 4.7B). WD-fed LOE mice showed reduced hepatic *Hmgcr* (Figure 4.7A) mRNA levels compared to chow-fed LOE mice, with a similar trend in *Hmgcr* mRNA expression on HF diet though this did not reach significance (Figure 4.7A).

LOE mice also showed reduced hepatic *Hmgcs* mRNA levels with WD feeding (Figure 4.7B). HSD1 KO mice displayed no significant differences in hepatic *Hmgcr* mRNA levels with HF and WD feeding (Figure 4.7A), though decreased levels of *Hmgcs* mRNA were observed with WD feeding (Figure 4.7B). A significant interaction between genotype and diet was observed on *Hmgcr* mRNA expression (Figure 4.7A) and, consistent with decreased hepatic *Srebp2* expression in chow-fed HSD1 KO mice, hepatic *Hmgcr* mRNA levels were reduced in chow-fed HSD1 KO mice compared to WT controls on the same diet (Figure 4.7A), though this was not observed previously (Morton *et al*., 2001).

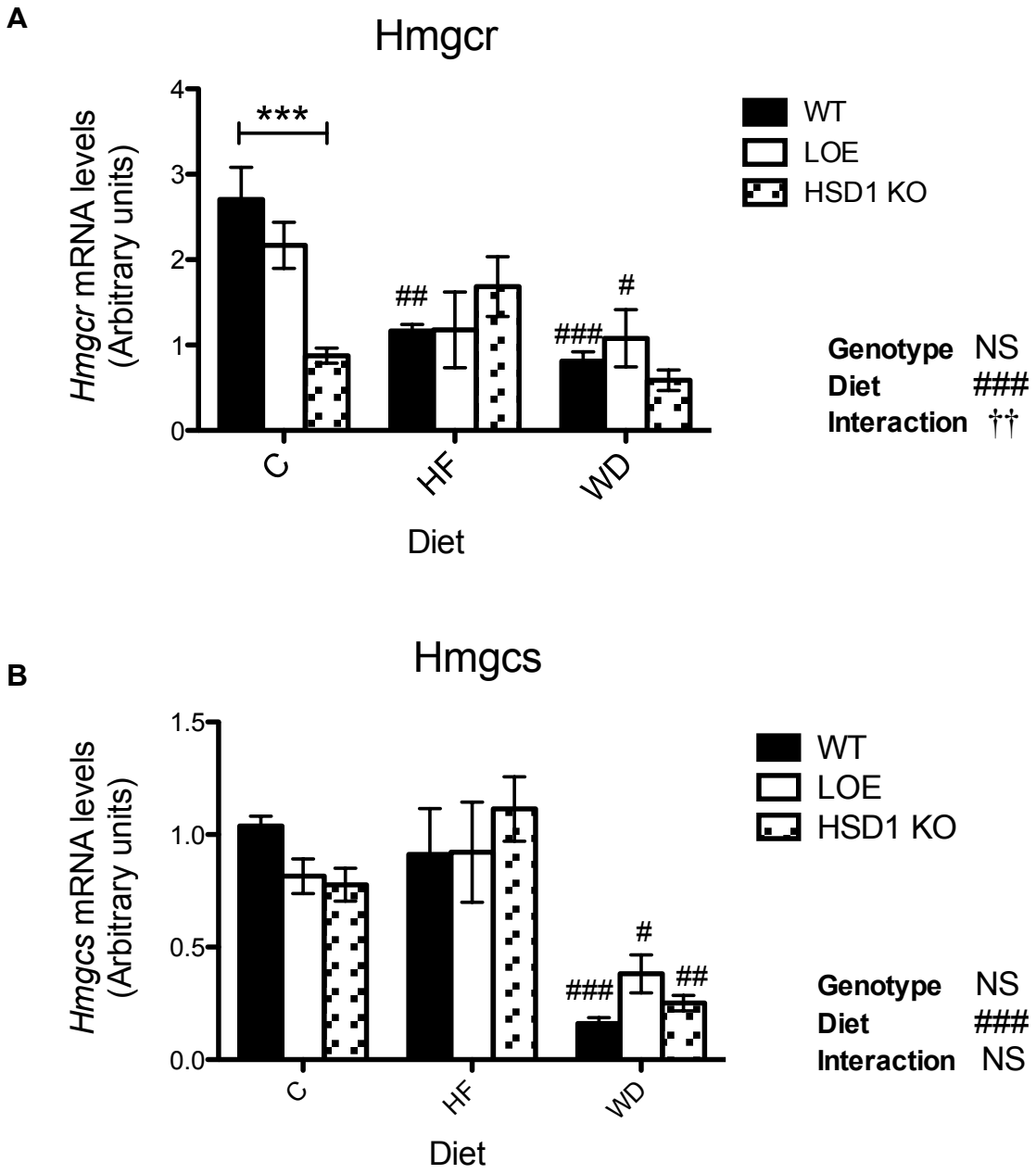


Figure 4.7: Significant effect of diet is observed on hepatic *Hmgcr* and *Hmgcs* mRNA levels.

Total RNA was extracted from livers of male wild-type control (WT), liver 11β-HSD1 overexpressor (LOE) and 11β-HSD1 knockout (HSD1 KO) mice fed one of the following diets for 12 weeks; chow (C), high fat (HF) or western diet (WD). **(A)** *Hmgcr,* and **(B)** *Hmgcs* mRNA levels were measured by qPCR, and are expressed relative to the average of *Hprt* and *Actb* mRNAs, used as internal controls. Data are means ± SEM and were analysed by two-way ANOVA with Tukey's multiple comparisons *post-hoc* test; n=6/group; *** $p<0.001$ for effect of genotype; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ for effect of diet; †† $p<0.01$ for interaction between genotype and diet; NS, not significant.

4.3.6. Increased hepatic *Lxrα* mRNA levels in WD-fed LOE mice compared to WD-fed WT controls

Consistent with its role as a sterol sensor that governs cholesterol absorption, transport and catabolism, LXRα is upregulated by cholesterol-rich and high fat diets (Janowski *et al*., 1996; Lehman *et al*., 1997; Peet *et al*., 1998; Zhang and Mangelsdorf, 2002). Hepatic *Lxrα* mRNA levels are increased in LOE mice (see 3.3.2; Paterson *et al*., 2004). As for 11β-HSD1 deficiency and LXRα expression, a recent study that used an antisense oligonucleotide to knockdown hepatic 11β-HSD1 in C57BL/6 mice showed no alteration in hepatic *Lxrα* mRNA levels with 11β-HSD1 knockdown (Li *et al*., 2011).

To test if LXRα expression was altered in liver of mice with 11β-HSD1 manipulations challenged with HF or cholesterol diets, *Lxrα* mRNA levels were measured in livers from male 17-18 week old WT, LOE, and HSD1 KO mice fed chow, HF diet or WD (for 12 weeks from 5-6 weeks of age).

A significant effect of genotype was observed on the levels of hepatic *Lxrα* mRNA such that WD-fed LOE mice showed increased levels of hepatic *Lxrα* mRNA compared to WD-fed WT mice (Figure 4.8). There was a trend for increased hepatic *Lxrα* mRNA levels in chow-fed LOE mice compared to chow-fed WT mice but this did not achieve significance (Figure 4.8), possibly because of low sample number. No effect of diet, or interaction between genotype and diet, were observed on hepatic *Lxrα* mRNA levels (Figure 4.8).

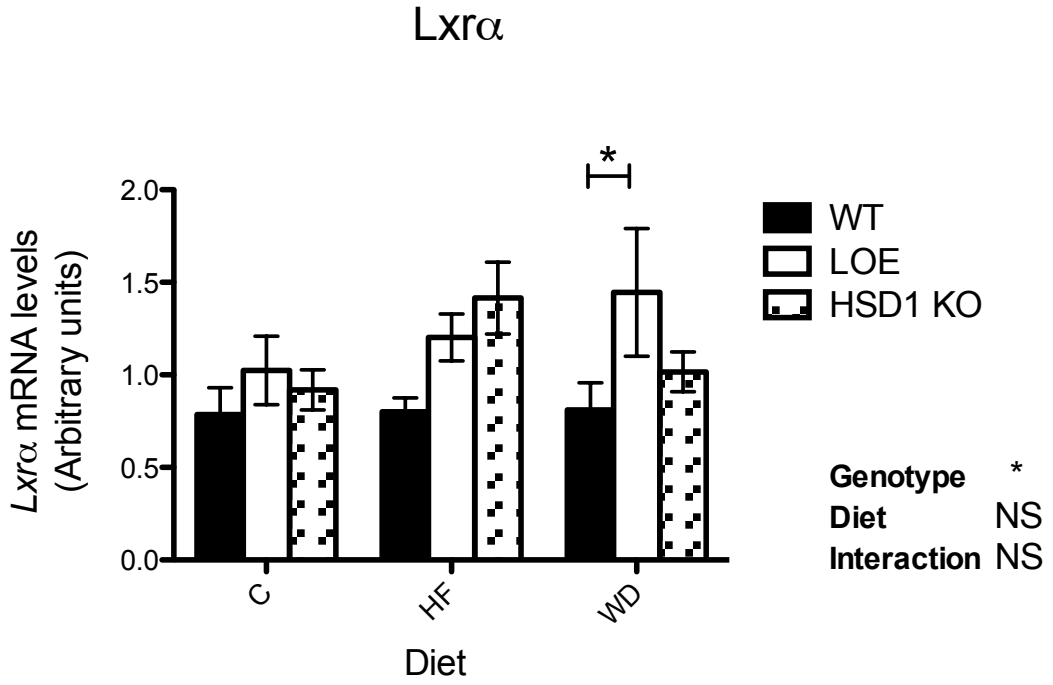


Figure 4.8: Increased hepatic *Lxrα* mRNA levels in WD-fed LOE mice compared to WT mice on the same diet.

Total RNA was extracted from livers of male wild-type control (WT), liver 11β-HSD1 overexpressor (LOE) and 11β-HSD1 knockout (HSD1 KO) mice fed one of the following diets for 12 weeks from 5-6 weeks of age; chow (C), high fat (HF) or western diet (WD). Hepatic *Lxrα* mRNA levels were measured by qPCR, and are expressed relative to the average of *Hprt* and *Actb* mRNAs, used as internal controls. Values are means ± SEM and were analysed by two-way ANOVA with Tukey's multiple comparisons *post-hoc* test; n=6/group; * $p<0.05$ for effect of genotype; NS, not significant.

4.3.7. No effect of genotype or diet on hepatic *Cyp7a1* mRNA levels

In rodents, CYP7A1 is upregulated in response to lipid and cholesterol feeding, via LXRα (Janowski *et al*., 1996; Lehmann *et al*., 1997; Peet *et al*., 1998; Zhang and Mangelsdorf, 2002). Hepatic *Cyp7a1* mRNA levels are reportedly increased with liver 11β-HSD1 overexpression (Paterson *et al*., 2004), although this was not observed in the current study (see 3.3.3). Hepatic *Cyp7a1* expression has not been examined in 11β-HSD1 deficient mice.

To establish whether hepatic induction of *Lxrα* in WD-fed LOE mice leads to increased clearance of cholesterol via bile acid synthesis, *Cyp7a1* mRNA levels were measured in the liver samples from male, 17-18 week old LOE mice following dietary intervention as described above. Additionally, hepatic *Cyp7a1* mRNA levels were measured in HSD1 KO mice to determine whether 11β-HSD1 deficiency altered cholesterol clearance through the CYP7A1 bile acid pathway. WT C57BL/6 mice served as controls.

There were no significant effects of genotype or diet, or interaction between the two, on hepatic *Cyp7a1* mRNA levels (Figure 4.9). These results suggest no significant changes in bile acid synthesis in either LOE or HSD1 KO mice compared with WT controls, on any of the diets examined.

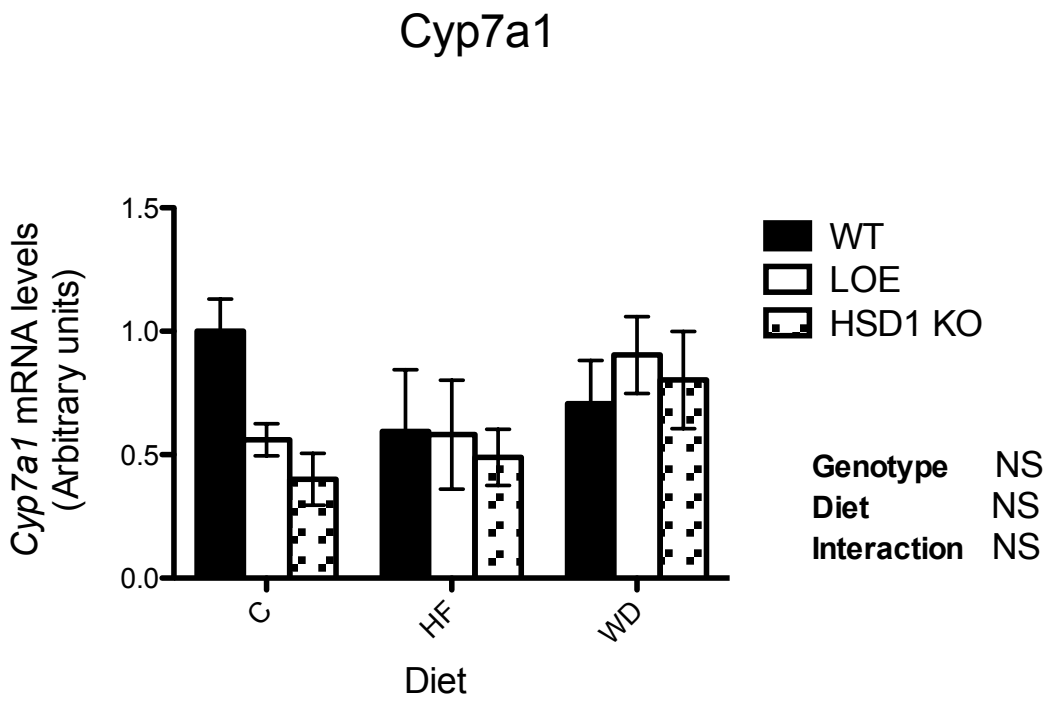


Figure 4.9: There is no effect of diet or genotype on hepatic *Cyp7a1* mRNA levels in LOE, WT and HSD1 KO mice fed Chow (C), High Fat (HF) or Western (WD) diet.

Total RNA was extracted from livers of male wild-type control (WT), liver 11β-HSD1 overexpressor (LOE) and 11β-HSD1 knockout (HSD1 KO) mice fed one of the following diets for 12 weeks from 5-6 weeks of age; chow (C), high fat (HF) or western diet (WD). Hepatic *Cyp7a1* mRNA levels were measured by qPCR, and are expressed relative to the average of *Hprt* and *Actb* mRNAs, used as internal controls. Values are means ± SEM and were analysed by two-way ANOVA; n=6/group; NS, not significant.

4.3.8. Significant effect of diet on hepatic *Cyp27a1* and *Cyp7b1* mRNA levels

Bile acid synthesis can occur through two pathways, the classical pathway or the alternative pathway (Ferdinandusse and Houten, 2006). Whilst the classical pathway starts with the rate-limiting 7-α hydroxylation of cholesterol by CYP7A1, the alternative pathway begins with the hydroxylation of the cholesterol side chain by sterol 27 hydroxylase (CYP27A1), followed by 7-α hydroxylation of the oxysterol intermediates by oxysterol 7-α hydroxylase (CYP7B1) (Wu *et al*., 1999; Norlin and Wikvall, 2007). Studies using mice in which CYP7A1, CYP27A1, or CYP7B1 was rendered non-functional have generated new insights into the quantitative contributions of the classic and alternate pathways in this species (Rosen *et al*., 1998; Schwarz *et al*., 1998; Li-Hawkins *et al*., 2000; Repa *et al*., 2000a). These as well as other studies carried out in rats suggest that the amount of total bile acid synthesis via the alternate pathway is significantly greater in these animal models (up to 25-30%) than appears to be the case in humans (6%) (Vlahcevic *et al*., 1997; Duane and Javitt, 1999).

To determine whether cholesterol elimination through bile acids is altered in HF- and WD-fed LOE and HSD1 KO mice via the alternative bile acid synthesis pathway, *Cyp27a1* and *Cyp7b1* mRNA levels were measured in liver samples from male 17-18 week old LOE and HSD1 KO mice at the end of this dietary experiment. As above, WT C57BL/6 mice served as controls.

A significant effect of diet was observed on hepatic *Cyp27a1* mRNA levels with WD-fed HSD1 KO mice having decreased *Cyp27a1* mRNA expression, compared to chow-fed HSD1 KO mice (Figure 4.10) Two-way ANOVA also showed a significant interaction between genotype and diet (Figure 4.10). HF-fed LOE mice exhibited reduced levels of hepatic *Cyp27a1* mRNA compared to WT mice on the same diet (Figure 4.10), suggesting decreased cholesterol elimination through the alternative bile acid synthesis pathway in HF-fed LOE mice relative to HF-fed WT controls.

Although the two-way ANOVA showed a significant effect of genotype as well as an interaction between genotype and diet on hepatic *Cyp7b1* mRNA levels (Figure 4.11),

the Tukey's *post-hoc* test did not detect genotype differences on any of the diets. Therefore, a Fisher's LSD *post-hoc* test was used. There were decreased hepatic *Cyp7b1* mRNA levels in HF-fed LOE and HSD1 KO mice compared to HF-fed WT mice (Figure 4.11). With respect to diet differences, WT mice showed increased hepatic *Cyp7b1* mRNA levels with HF feeding, and LOE mice exhibited reduced levels of *Cyp7b1* mRNA with WD feeding (Figure 4.11).

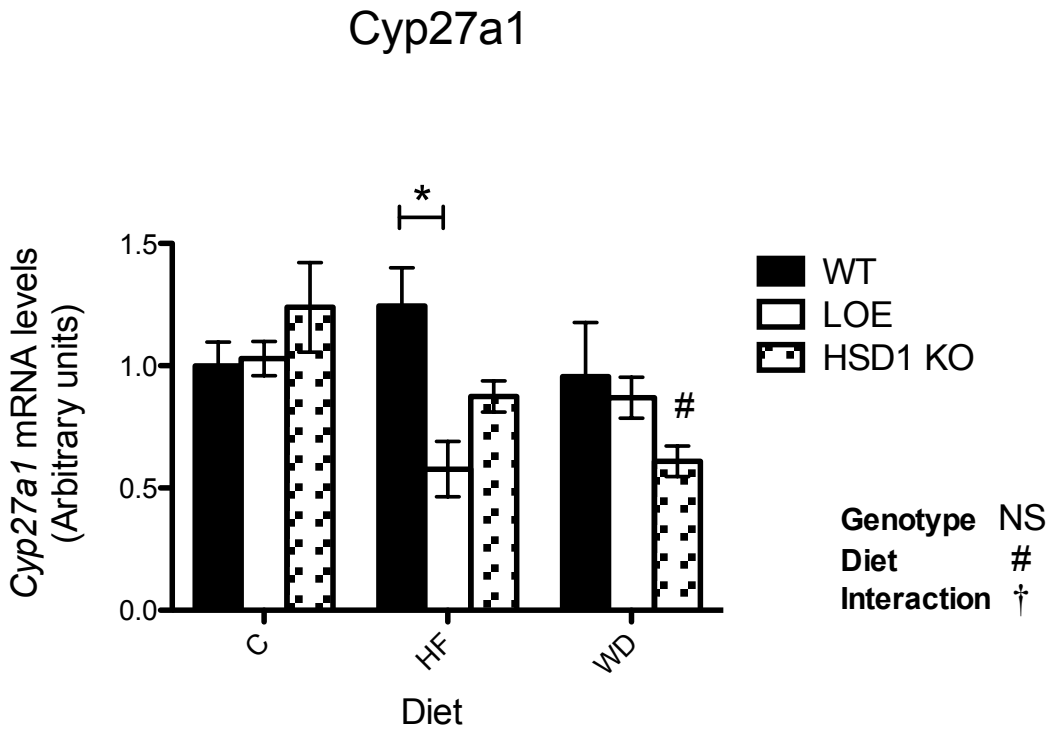


Figure 4.10: There is an effect of diet on hepatic *Cyp27a1* mRNA levels.

Total RNA was extracted from livers of male wild-type control (WT), liver 11β-HSD1 overexpressor (LOE) and 11β-HSD1 knockout (HSD1 KO) mice fed one of the following diets for 12 weeks; chow (C), high fat (HF) or western diet (WD). Hepatic *Cyp27a1* mRNA levels were measured by qPCR, and are expressed relative to the average of *Hprt* and *Actb* mRNAs, used as internal controls. Values are means ± SEM and were analysed by two-way ANOVA with Tukey's multiple comparisons *post-hoc* test; n=6/group; * $p<0.05$ for effect of genotype; # $p<0.05$ for effect of diet; † $p<0.05$ for interaction between genotype and diet; NS, not significant.

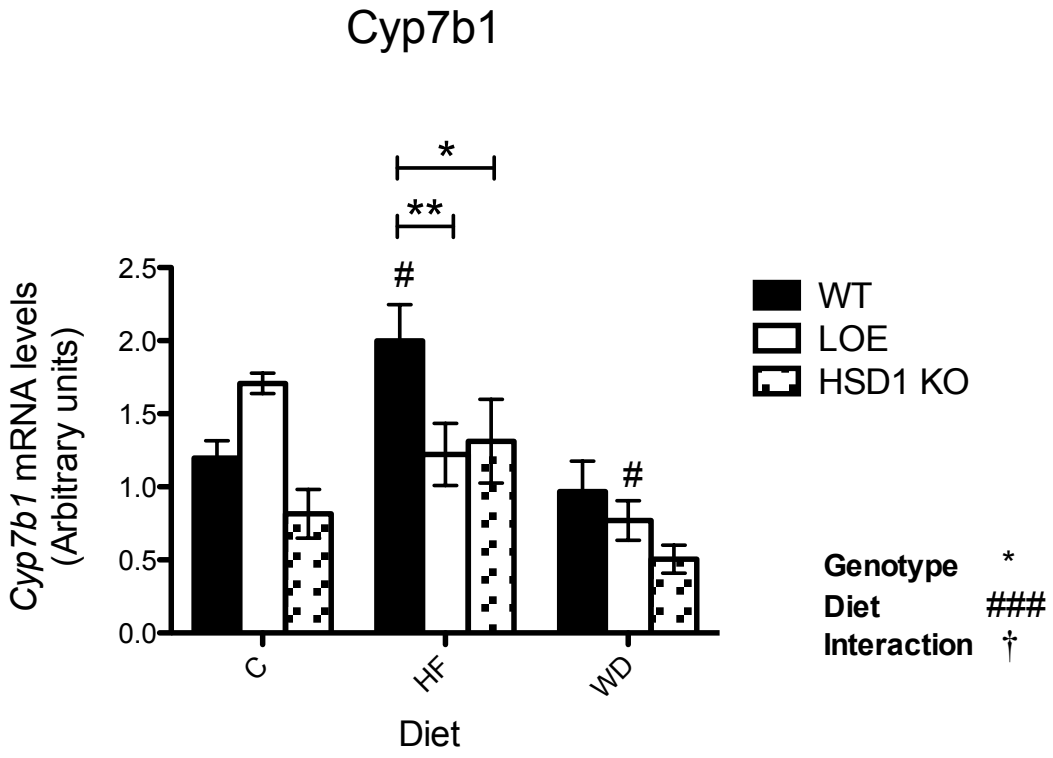


Figure 4.11: Significant effects of diet and genotype are observed on hepatic *Cyp7b1* mRNA levels.

Total RNA was extracted from livers of male wild-type control (WT), liver 11β-HSD1 overexpressor (LOE) and 11β-HSD1 knockout (HSD1 KO) mice fed one of the following diets for 12 weeks from 5-6 weeks of age; chow (C), high fat (HF) or western diet (WD). Hepatic *Cyp7b1* mRNA levels were measured by qPCR, and are expressed relative to the average of *Hprt* and *Actb* mRNAs, used as internal controls. Values are means ± SEM and were analysed by two-way ANOVA with Fisher's LSD *post-hoc* test; n=6/group; * $p<0.05$, ** $p<0.01$ for effect of genotype; # $p<0.05$, ### $p<0.001$ for effect of diet; † $p<0.05$ for interaction between genotype and diet.

4.3.9. Increased hepatic *Abcg5* and *Abcg8* mRNA levels in WD-fed LOE mice compared to WD-fed WT mice

As mentioned previously, *in vivo* and *in vitro* experiments have shown that LXR promotes cholesterol elimination by upregulating transcription of *Abcg5/8* in the liver where these ABC transporters promote cholesterol excretion into bile (Berge *et al*., 2000; Repa *et al*., 2002; Graf *et al*., 2003; Yu *et al*., 2004; Yu *et al*., 2014; see 3.3.4), as well as activating transcription of intestinal *Abcg5/8,* where the transporters act to limit cholesterol absorption from the gut (Zhang and Mangelsdorf, 2002; Geyeregger *et al*., 2006; Yu *et al*., 2014). Consistent with a role in preventing excess dietary sterol accumulation, levels of *Abcg5/8* mRNAs increase in both liver and intestine in response to cholesterol-rich diets (Su *et al*., 2012). Hepatic *Abcg5* and *Abcg8* mRNA levels were increased with liver 11β-HSD1 overexpression (see 3.3.4), and antisense-mediated inhibition of 11β-HSD1 in C57BL/6 mice reduced hepatic *Abcg5/8* expression compared with controls (Li *et al*., 2011). Hepatic *Abcg5/8* expression has not been investigated in HF- and WD-fed LOE or HSD1 KO mice.

To determine whether hepatic induction of *Lxrα* in WD-fed LOE mice potentially leads to increased hepatobiliary cholesterol secretion, and additionally to investigate effects of 11β-HSD1 deficiency combined with HF and WD feeding upon hepatobiliary cholesterol secretion, *Abcg5* and *Abcg8* mRNA levels were measured in livers of LOE and HSD1 KO mice following dietary intervention. WT C57BL/6 mice served as controls.

A significant effect of diet was observed on the levels of hepatic *Abcg5/8* mRNAs such that LOE mice showed significantly elevated levels of hepatic *Abcg5* (Figure 4.12A) and *Abcg8* (Figure 4.12B) mRNA with WD feeding. There were also increased hepatic *Abcg5* (Figure 4.12A) and *Abcg8* (Figure 4.12B) levels in WD-fed HSD1 KO and WT mice compared with chow feeding. A significant effect of genotype as well as interaction between genotype and diet were observed (Figure 4.12), and consistent with increased hepatic *Lxrα* expression in WD-fed LOE mice, hepatic *Abcg5* (Figure 4.12A) and *Abcg8* (Figure 4.12B) mRNA levels were elevated in WD-fed LOE mice compared to WT controls on the same diet.

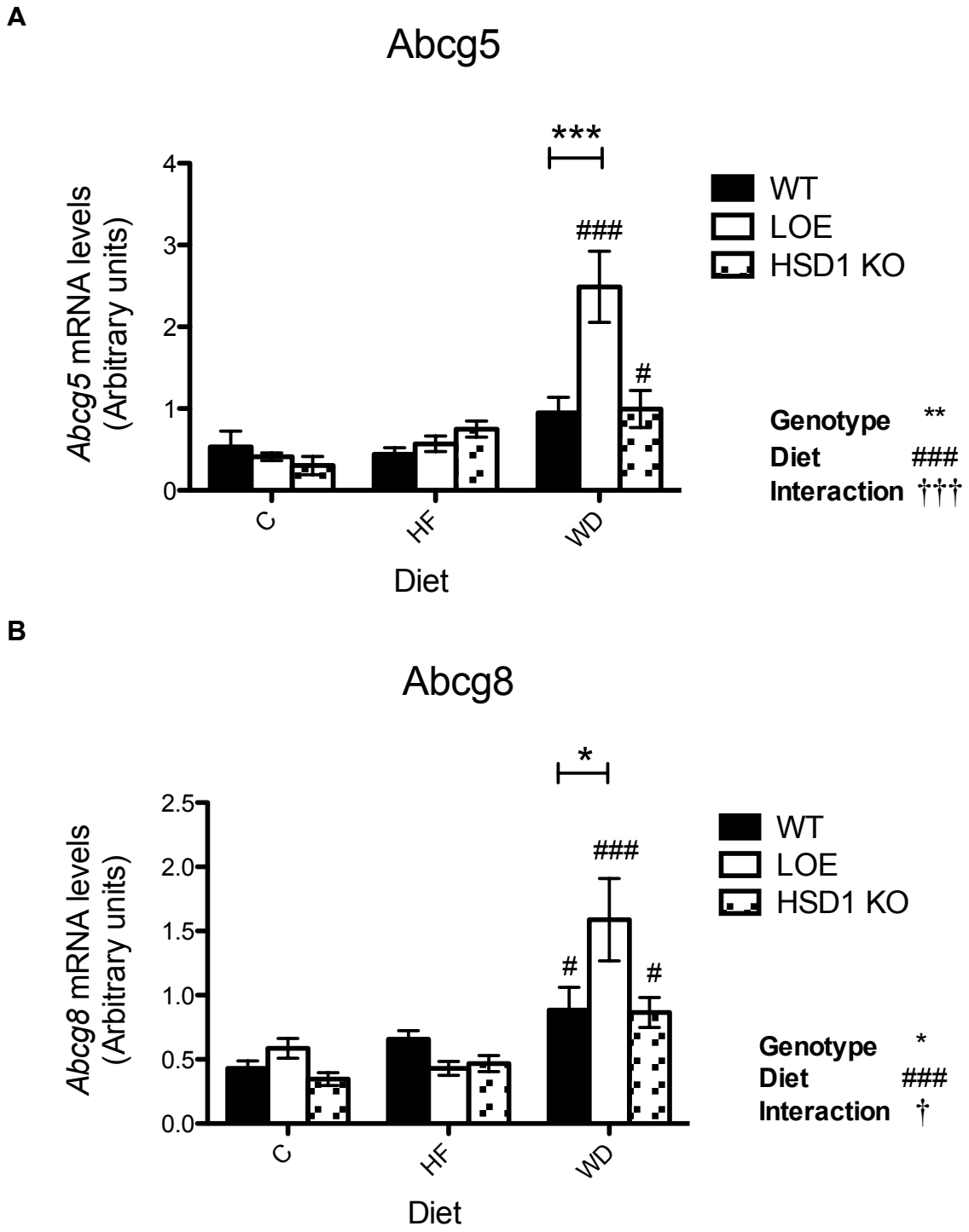


Figure 4.12: Hepatic *Abcg5* and *Abcg8* mRNA are increased in LOE mice fed WD compared to WT mice on same diet.

Total RNA was extracted from livers of male wild-type control (WT), liver 11β-HSD1 overexpressor (LOE) and 11β-HSD1 knockout (HSD1 KO) mice fed one of the following diets for 12 weeks; chow (C), high fat (HF) or western diet (WD). **(A)** *Abcg5,* and **(B)** *Abcg8* mRNA levels were measured by qPCR, and are expressed relative to the average of *Hprt* and *Actb* mRNAs. Values are means ± SEM and were analysed by two-way ANOVA with Tukey's multiple comparisons *post-hoc* test; n=6/group; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ for effect of genotype; # $p<0.05$, ### $p<0.001$ for effect of diet; † $p<0.05$, ††† $p<0.001$ for interaction between genotype and diet.

4.3.10. Significant effect of diet on hepatic *Apoe* mRNA levels

Apolipoprotein E (ApoE) is a principal component of chylomicron remnants and VLDL, and is involved in their hepatic uptake. ApoE is essential for the catabolism of these TG-rich lipoprotein constituents (Plump *et al*., 1992; Zhang *et al*., 1992). Unlike most of the apolipoproteins that are mainly synthesised in the liver and intestine, ApoE is also synthesised in macrophages and adipocytes (Curtiss and Boisvert, 2000; Mazzone, 1996). In these cells, ApoE facilitates cholesterol efflux in both humans and mice (Zhang *et a*l., 1996; Langer *et al*., 2000; Curtiss and Boisvert, 2000). *Apoe* gene expression is directly regulated by LXR in both macrophages and adipocytes (Laffitte *et al*., 2001).

To investigate whether hepatic induction of *Lxrα* in WD-fed LOE mice is associated with increased hepatic ApoE expression, *Apoe* mRNA levels were measured in livers of mice from the dietary experiment described above.

A significant effect of diet on hepatic *Apoe* mRNA levels was observed such that WD-fed HSD1 KO mice showed decreased hepatic *Apoe* mRNA levels compared to chow-fed HSD1 KO mice (Figure 4.13).

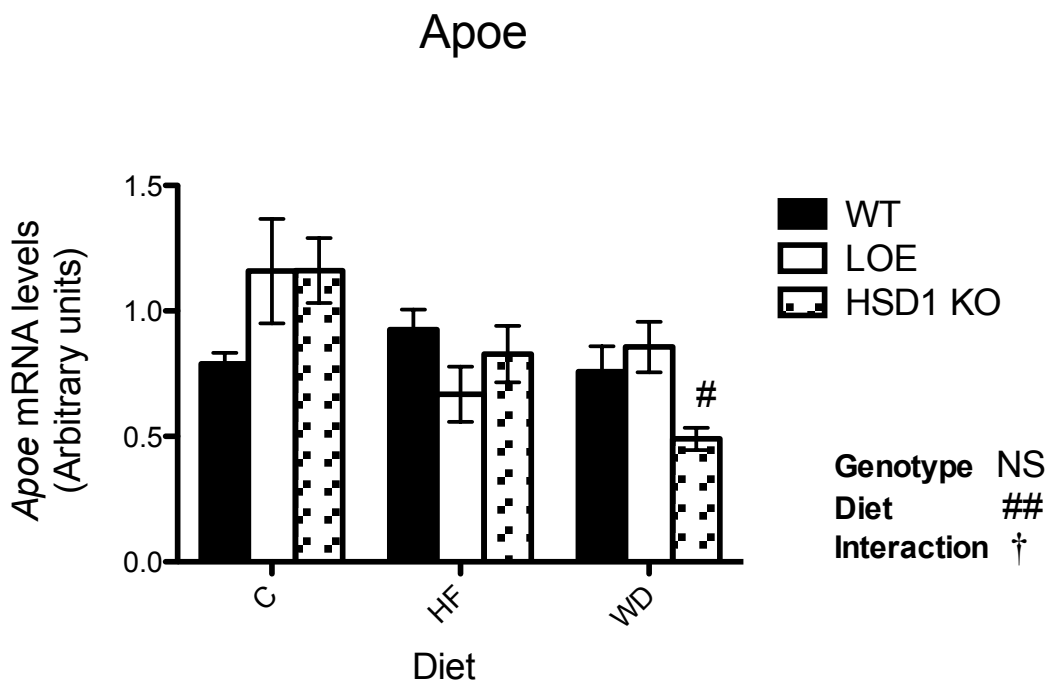


Figure 4.13: Significant effect of diet is observed on hepatic *Apoe* mRNA levels.

Total RNA was extracted from livers of male wild-type control (WT), liver 11β-HSD1 overexpressor (LOE) and 11β-HSD1 knockout (HSD1 KO) mice fed one of the following diets for 12 weeks from 5-6 weeks of age; chow (C), high fat (HF) or western diet (WD). Hepatic *Apoe* mRNA levels were measured by qPCR, and are expressed relative to the average of *Hprt* and *Actb* mRNAs, used as internal controls. Values are means ± SEM and were analysed by two-way ANOVA with Tukey's multiple comparisons *post-hoc* test; n=6/group; # $p<0.05$, ## $p<0.01$ for effect of diet; † $p<0.05$ for interaction between genotype and diet; NS, not significant.

4.3.11. Significant effect of diet on hepatic *ApoA1* mRNA levels

Apolipoprotein A1 (ApoA1) is the major protein component of the HDL particle (Barter and Rye, 1996) and its concentrations are closely linked with plasma HDL (Rubin *et al*., 1991; Williamson *et al*., 1992). ApoA1 is secreted mostly by the liver and plays an important role in RCT (Plump *et al*., 1994).

To investigate if ApoA1 expression is altered in livers of mice with 11β-HSD1 manipulations that were challenged with HF or cholesterol diets, hepatic *ApoA1* mRNA levels were measured in livers of LOE and HSD1 KO mice following dietary intervention as described above. WT C57BL/6 mice served as controls.

Two-way ANOVA showed a significant effect of diet, as well as a significant interaction between genotype and diet, on hepatic *ApoA1* mRNA levels (Figure 4.14). However, the Tukey's *post-doc* test did not detect diet differences, or any differences between genotypes on the diets investigated. Carrying out a Fisher's LSD test revealed significantly decreased hepatic *ApoA1* mRNA levels in LOE and HSD1 KO mice with WD-feeding (Figure 4.14). Also, there was a reduction in hepatic *ApoA1* mRNA levels in WD-fed LOE and HSD1 KO mice compared to WD-fed WT mice (Figure 4.14).

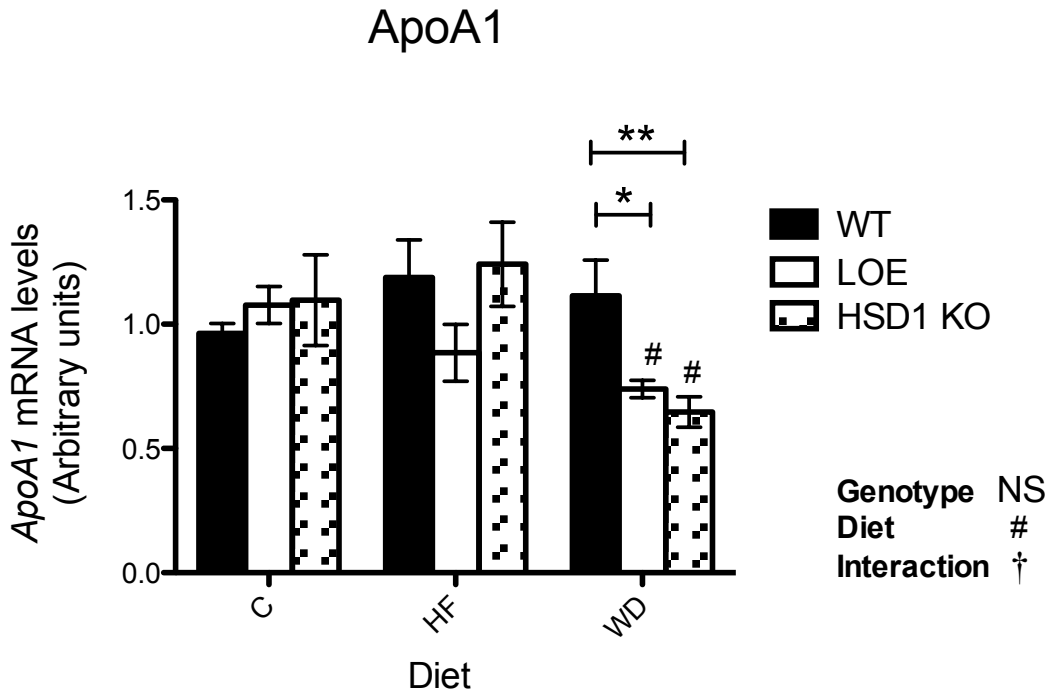


Figure 4.14: Significant effect of diet is observed on hepatic *ApoA1* mRNA levels.

Total RNA was extracted from livers of male wild-type control (WT), liver 11β-HSD1 overexpressor (LOE) and 11β-HSD1 knockout (HSD1 KO) mice fed one of the following diets for 12 weeks; chow (C), high fat (HF) or western diet (WD). Hepatic *ApoA1* mRNA levels were measured by qPCR, and are expressed relative to the average of *Hprt* and *Actb* mRNAs, used as internal controls. Values are means ± SEM and were analysed by two-way ANOVA with Fisher's LSD *post-hoc* test; n=6/group; * $p<0.05$, ** $p<0.01$ for effect of genotype; # $p<0.05$ for effect of diet; † $p<0.05$ for interaction between genotype and diet; NS, not significant.

4.4. Discussion

The work in this chapter was designed to test the hypothesis that liver 11β-HSD1 overexpression, (through its role in the metabolism of 7-oxysterols), combined with cholesterol/fat feeding, promotes hepatic cholesterol synthesis via hepatic induction of SREBP-2 target genes involved in the cholesterol biosynthetic pathway, whereas 11β-HSD1 deficiency leads to the opposite effect.

Contrary to predictions, liver 11β-HSD1 overexpression failed to support a role in hepatic cholesterol synthesis, both on WD and HF diet. Unexpectedly, chow-fed LOE mice showed reduced hepatic *Srebp2* mRNA levels compared to chow-fed WT mice, and with WD feeding, both WT and LOE mice reduced hepatic *Srebp2* mRNA expression compared to chow diet. Furthermore, there were no significant differences in the hepatic mRNA levels of *Hmgcr* and *Hmgcs* between WT and LOE mice on any of the diets, consistent with findings from chow-fed, 6-8 week old LOE mice (Chapter 3). There are two sources of cholesterol that circulate in plasma; cholesterol derived from the diet, and cholesterol that is mainly synthesised by the liver. In animals on a cholesterol-free diet, the only source of plasma cholesterol is *de novo* cholesterol biosynthesis, whereas when dietary cholesterol is available/plentiful, feedback inhibition of cholesterol biosynthesis occurs so that very little plasma cholesterol is derived from *de novo* synthesis (Gould, 1951; Siperstein, 1970; Brown and Goldstein, 1997; Edwards *et al*., 2000; Maxwell *et al*., 2003). Consequently, numerous studies have shown that cholesterol-rich diets lead to downregulation of genes encoding hepatic cholesterol biosynthetic enzymes, including HMGCR (Maxwell *et al*., 2003; Mutungi *et al*., 2007; Boone *et al*., 2011) and HMGCS (Kushwaha *et al*., 1995; Boone *et al*., 2011). Effects of HF feeding upon cholesterol synthesis are not as clear; whilst some studies demonstrate downregulation of *Hmgcr* expression and enzyme activity with HF feeding (Rudling *et al*., 1992; Sumiyoshi *et al*., 2006), others show that HF feeding upregulates HMGCR expression (mRNA and protein as well as enzyme activity) and increases cholesterol synthesis (Lin *et al*., 2005; Jensen *et al*., 2012; Wu *et al*., 2013). In the current study, WT mice showed the expected dietary effects in that levels of *Hmgcr* mRNA were reduced in the liver with HF or WD feeding, and hepatic *Hmgcs* mRNA levels were decreased with cholesterol-rich WD.

However, contrary to the hypothesis, LOE mice behaved like WT mice on the cholesterol-rich WD (and also on HF diet to an extent) with respect to hepatic *Hmgcr* and *Hmgcs* mRNA expression. These results imply that LOE mice show a normal physiological response with respect to regulation of cholesterol synthetic enzymes when faced with high fat or cholesterol dietary challenges.

Interestingly, chow-fed HSD1 KO mice exhibited decreased hepatic *Hmgcr* levels compared to WT mice on the same diet, suggesting lower levels of basal cholesterol synthesis with 11β-HSD1 deficiency. Because GCs inhibit cholesterol biosynthesis (Melnykovych *et al*., 1976; Russell, 1992; Russell, 2003), a possible mechanism to explain this may be that 11β-HSD1 deficiency results in elevated levels of 7-KC (through lack of conversion to 7β-HC), which provides an endogenous brake on cholesterol biosynthesis (Brown *et al*., 2002). The above-mentioned finding is not consistent with a previous study that showed no alteration in hepatic *Hmgcr* mRNA levels with 11β-HSD1 deficiency (Morton *et al*., 2001). A possible reason for this discrepancy is that Morton *et al*. (2001) used HSD1 KO mice on the original MF1/129 background in their study (Morton *et al*., 2001), whereas the current study used HSD1 KO mice that have been rederived onto the C57BL/6 strain.

Cholesterol conversion to bile acids in the liver and their subsequent faecal excretion represents a major route for cholesterol elimination from the body (Arias *et al*., 2009; Zhang *et al*., 2012). LXRα, a nuclear receptor that is upregulated by cholesterol-rich diets, is key in governing cholesterol catabolism through activation of transcription of hepatic *Cyp7a1* (in rodents at least), the gene that encodes the rate-limiting enzyme of the classic bile acid synthesis pathway (Janowski *et al*., 1996; Lehman *et al*., 1997; Peet *et al*., 1998; Zhang and Mangelsdorf, 2002; Nomiyama and Bruemmer, 2008; Hong *et al*., 2012). In this study, hepatic *Lxrα* mRNA expression was unaffected by diet in WT and HSD1 KO mice (and did not significantly differ in chow-fed mice between genotypes), but was increased in WD-fed LOE mice. Despite this upregulation in hepatic *Lxrα* mRNA levels of WD-LOE mice, there was no evidence of increased *Cyp7a1* mRNA expression in livers of WD-LOE mice. There were also no differences in *Cyp7a1* mRNA levels between WD-fed WT and HSD1 KO mice, or in HF-fed mice between genotypes. Taken together, these results suggest no change in

bile acid synthesis in either LOE or HSD1 KO mice with HF and WD feeding. With respect to WT mice, these results contradict a previous study by Gnerre *et al*. (2005) who observed elevated hepatic *Cyp7a1* mRNA levels in C57BL/6 mice fed a chow diet that was supplemented with 2% cholesterol (Gnerre *et al*., 2005). However, it is important to point out that the WD used in this study contained only 0.2% cholesterol (a more physiologically relevant level), which was substantially lower than the diet used by Gnerre and coworkers. It is possible that the WD used in this study results in cholesterol excess that is enough to decrease cholesterol biosynthesis as demonstrated by reduced hepatic *Hmgcr* and *Hmgcs* mRNA on WD-fed mice, but not high enough to increase bile acid synthesis. Also, gender-related effects on bile acid synthesis in various species are well-documented. For example, CYP7A1 activity is higher in females in both rats and mice (Gielen *et al*., 1976; Schwarz *et al*., 2001).

In addition to the regulation of bile acid synthesis, LXR also regulates cholesterol excretion from hepatocytes into the bile, and this depends on the ability of LXR to activate hepatic *Abcg5* and *Abcg8* transcription, thereby promoting cholesterol excretion into bile (Repa *et al*., 2002; Yu *et al*., 2002). Consistent with these studies, and with the observed LXRα upregulation in WD-fed LOE mice in the current study, hepatic *Abcg5* and *Abcg8* mRNA levels were elevated in WD-fed LOE mice, suggesting increased hepatobiliary cholesterol secretion in these mice on a cholesterol-rich diet. These findings corroborate results from chow-fed, 6-8 week old LOE mice (Chapter 3).

Apoe is another gene directly regulated by LXR that is involved in RCT, and therefore important for cholesterol homeostasis. No genotype effects were observed on hepatic *Apoe* mRNA levels in this study. Nevertheless, this does not exclude that induction of LXRα in LOE mice may activate expression of *Apoe* in macrophages and adipose tissue, as previous reports have highlighted tissue-specific regulation of *Apoe* by LXR (Laffitte *et al*., 2001; Beyea *et al*., 2006).

Previously, feeding a chow diet supplemented with 2% cholesterol to mice has shown to upregulate hepatic *Apoe* expression (Peet *et al*., 1998). However, in the current study, WD-fed HSD1 KO mice showed reduced hepatic *Apoe* mRNA levels

compared to chow-fed HSD1 KO mice. Although in macrophages, ApoE is known to facilitate RCT (Zhang *et al*., 1996; Hara *et al*., 2003), in hepatocytes, ApoE enhances the production of VLDL particles (Tsukamoto *et al*., 2000). Increased hepatic VLDL production leads to accumulation of atherogenic lipoproteins and TG levels in the circulation, and is a common characteristic of hyperlipidaemia as well as type 2 diabetes (Huang *et al*., 1998; Tsukamoto *et al*., 2000; Kurano *et al*., 2011). HSD1 KO mice (chow-fed) have previously been shown to display reduced plasma TG levels compared to WT mice (Morton *et al*., 2001). Decreased hepatic *Apoe* mRNA levels in WD-fed HSD1 KO mice in this study may aid in keeping total lipid and lipoprotein levels in check.

It is worth noting that there are slight discrepancies between the gene expression data from 6-8 week old chow-fed WT/LOE mice in Chapter 3 and 5-6 week old chow-fed WT/LOE mice in this experiment. Chow-fed LOE mice in this study revealed reduced hepatic *Srebp2* mRNA levels compared to chow-fed WT mice although no such difference was observed in Chapter 3. Also, in this experiment, chow-fed LOE mice revealed a trend (although insignificant) for decreased hepatic *Cyp7a1* mRNA levels compared to chow-fed WT mice, whereas there were clearly no differences in hepatic *Cyp7a1* mRNA levels between chow-fed WT and LOE mice in Chapter 3. The reasons for these inconsistencies are unclear, but factors such as carrying out these studies at different times or on mice of different age might contribute to the above-mentioned discrepancies.

This study has several limitations and future work should address these issues; for instance, total cholesterol levels and cholesterol distribution profiles were not investigated, and protein/enzyme activity levels for HMGCR and HMGCS were not measured. It is fascinating that chow-fed HSD1 KO mice showed increased body weight gain compared to chow-fed WT mice. Food intake per mouse was not measured and mice were housed in groups of 5-6 per cage, making it impossible to assess the amount of diet consumed by each mouse. Furthermore, this study cannot fully distinguish whether the effects observed are dependent on metabolism of GCs or of oxysterols by 11β-HSD1 and requires further investigation in adrenalectomised mouse models (which have endogenous GCs removed). Also, liver and plasma

samples of the mouse models in this dietary study were sent to Professor William Griffith's laboratory (Swansea University) for determination of oxysterol levels, but the results were not returned in time for inclusion in this thesis.

In summary, the data in this chapter do not support a role for hepatic 11β-HSD1 in *de novo* cholesterol synthesis, and therefore, stand contrary to the hypothesis. LOE mice appear to 'sense' intracellular cholesterol excess and respond to it by activation of hepatobiliary cholesterol excretion demonstrated by increased hepatic *Abcg5* and *Abcg8* expression in WD-LOE mice. This may be mediated through increased hepatic *Lxrα* expression in these mice. Taken together, these results suggest a role for increased hepatic 11β-HSD1 in promoting biliary cholesterol secretion.

Chapter 5. Generation and Characterisation of Liver-specific 11β-HSD1 Knockout Mice

5.1. Introduction

As described in Chapters 1, 3 and 4, studies carried out in globally *Hsd11b1* deficient mice have shown that 11β-HSD1 deficiency improves metabolic outcome in obesity and is atheroprotective (Kotelevtsev *et al*., 1997; Morton *et al*., 2001; Morton *et al*., 2004; Hermanowski-Vosatka *et al*., 2005; Wamil *et al*., 2011; Kipari *et al*., 2013; see 1.4.1). Given the wide range of 11β-HSD1 expression (Tannin *et al*., 1991; Whorwood *et al*., 1993; Rajan *et al*., 1995; Tomlinson *et al.*, 2004), the contribution of different tissues to the improved metabolic outcome and atheroprotection observed with global deficiency of 11β-HSD1 remains unknown. However, liver is the major site of 11β-HSD1 expression (Agarwal *et al*., 1989; Tannin *et al*., 1991; Rajan *et al*., 1995; Tomlinson *et al*., 2004). Furthermore, at the start of this PhD study, the hypothesis (see 1.9 and 3.1) predicted that elevated hepatic 7-KC and decreased mature SREBP-2 levels would result from knockout of hepatocyte 11β-HSD1, suggesting decreased hepatic cholesterol synthesis. Findings from the previous chapter (Chapter 4, see Figure 4.7A) support this as chow-fed HSD1 KO mice showed reduced hepatic *Hmgcr* mRNA levels compared to WT mice on the same diet.

Unlike the model for hepatic overexpression of 11β-HSD1 (LOE mice), there was no existing *in vivo* model for hepatic 11β-HSD1 deficiency when this study commenced. To determine the effect(s) of hepatic 11β-HSD1 deficiency on cholesterol homeostasis as well as assess the importance of hepatic 11β-HSD1 in metabolic syndrome, a Cre-loxP genetic approach was employed to generate a mouse model of 11β-HSD1 deficiency specifically within the liver by using albumin (Alb) promoter-driven Cre recombination.

Albumin (encoded by *Alb*) is a soluble, monomeric protein that comprises about one-half of the blood serum protein (Gosling, 1995), and its synthesis is restricted to the liver (Miller *et al*., 1951; Lundsgaard-Hansen, 1986). *Alb-Cre* mice have been useful in studies involving Cre-dependent excision of loxP-flanked ("floxed") sequences in adult hepatocytes (Postic *et al*., 1999). Moreover, *Alb-Cre* transgenic mice have been shown to achieve liver-specific deletions with extremely high efficiency of

recombination (90% - 100%) (Gu *et al*., 2003; Fan *et al*., 2009). Breeding of *Alb-Cre* mice with in-house *Hsd11b1*$^{flox/flox}$ mice, which have exon 3 of the *Hsd11b1* gene flanked by *loxP* sites, should generate liver-specific 11β-HSD1 knockout mice. This chapter describes the generation of these knockout mice as well as their characterisation.

5.2. Hypothesis and Aims

Hypothesis: Liver 11β-HSD1 deficiency reduces hepatic cholesterol synthesis via downregulation of hepatic SREBP-2 and its target genes in the cholesterol biosynthetic pathway.

Aims

1) To generate mice with liver-specific *Hsd11b1* deletion (LKO mice).

2) To determine if LKO mice have *Hsd11b1* deleted specifically only in their livers.

3) To characterise the effects of liver 11β-HSD1 deficiency on metabolic parameters such as body weight, tissue weights, and expression of genes involved in cholesterol biosynthesis as well as cholesterol excretion/degradation.

5.3. Results

5.3.1. Reduced *Hsd11b1* mRNA levels as well as decreased 11β-HSD1 protein and enzyme activity levels in livers of LKO mice

Previously, a conditional *Hsd11b1* allele was generated by flanking exon 3 with *loxP* sites, and from this global 11β-HSD1 deficient mice were derived (unpublished). To generate liver-specific 11β-HSD1 knockout (LKO) mice, floxed homozygous *Hsd11b1* mice (*Hsd11b1*$^{flox/flox}$) on a C57BL/6 background were crossed with *Alb-Cre* transgenic mice (see 2.2.1 for further details), also on a C57BL/6 background, targeting Cre expression to hepatocytes (Postic *et al*., 1999). This generated mice with liver-specific deletion of *Hsd11b1*. As expected, the line of LKO mice was indistinguishable from floxed *Hsd11b1* controls with respect to fertility, gestation length and sex ratio.

To determine whether *Hsd11b1* mRNA levels are reduced/knocked out, as predicted, in livers of LKO mice, total RNA was extracted from frozen liver tissue of chow-fed, male 10-week-old LKO and their littermate control floxed mice. Hepatic *Hsd11b1* mRNA levels were measured via qPCR. To establish whether the predicted reduction in hepatic *Hsd11b1* mRNA levels is associated with decreased 11β-HSD1 protein levels as well as reduced 11β-HSD1 enzyme activity in liver, western blot analyses and enzyme activity assays were conducted, respectively.

Compared to littermate control floxed *Hsd11b1* mice, LKO mice showed undetectable hepatic *Hsd11bl* mRNA levels (Figure 5.1) with hepatic 11β-HSD1 protein levels being similarly negligible (Figure 5.2). Hepatic 11β-HSD1 enzyme activity was measured using TLC as well as HPLC. In the TLC experiment, 11β-HSD1 enzyme activity was reduced by ~80% in livers of LKO mice compared with livers of control mice (n=5/group; Figure 5.3). In the HPLC experiment, 11β-HSD1 enzyme activity was decreased by 88% in livers of LKO mice compared with those of control mice (n=6/group; Figure 5.4).

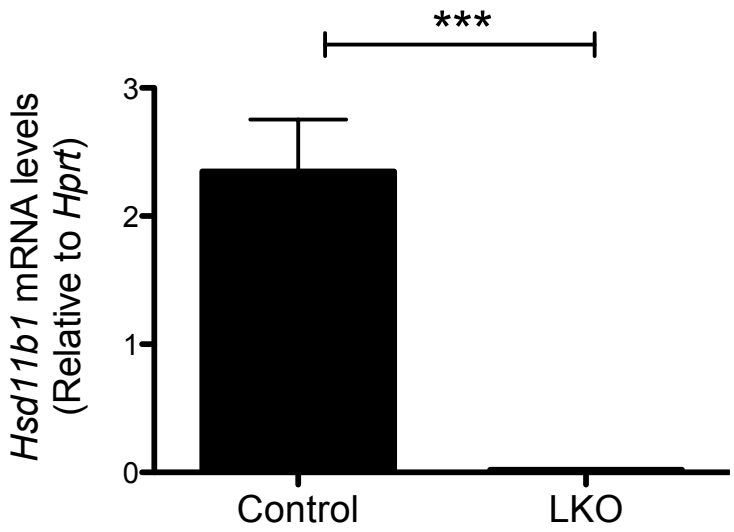


Figure 5.1: Levels of hepatic *Hsd11b1* mRNA are undetectable in LKO mice compared to their wild-type littermates.

Total RNA was extracted from livers of male chow-fed, 10 week old liver 11β-HSD1 knockout (LKO) mice and their floxed *Hsd11b1* littermates (Control). *Hsd11b1* mRNA levels were measured by qPCR and are expressed relative to *Hprt* mRNA levels, used as an internal control. Data are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=8-10/group; *** $p<0.001$.

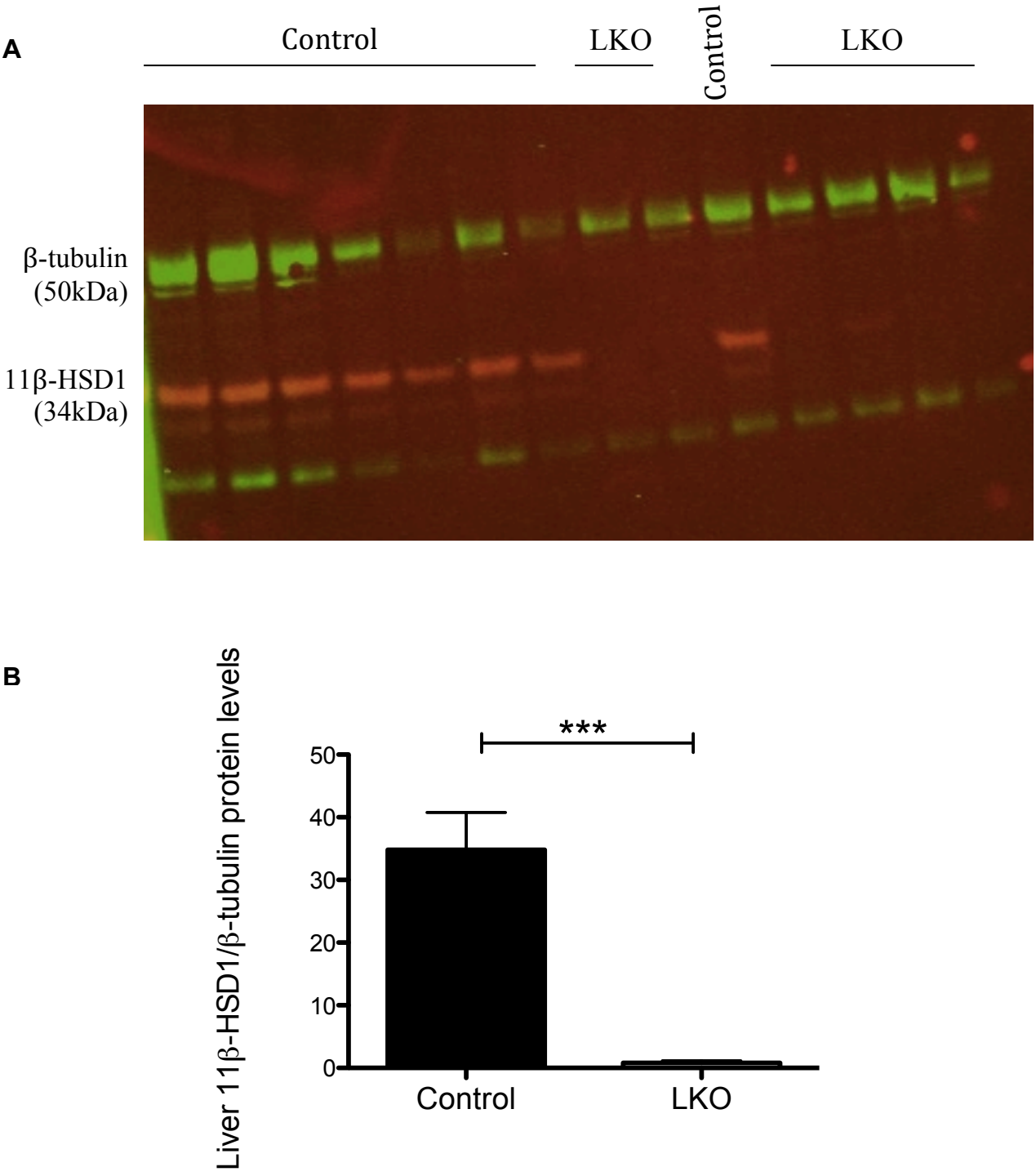


Figure 5.2: Hepatic 11β-HSD1 protein levels are negligible in LKO mice compared to their wild-type littermates.

Western blotting was used to examine 11β-HSD1 protein levels in liver samples from male, chow-fed, 10 week old liver-specific 11β-HSD1 knockout (LKO) mice and their floxed *Hsd11bl* littermates (Control). **(A)** Image of a representative western blot. Each lane contains 25μg protein from one mouse, appropriately labelled. β-tubulin was used as a loading control. **(B)** Density of bands in Figure 5.2A were quantified and plotted into a graph. Data are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=6-8/group; *** $p<0.0001$.

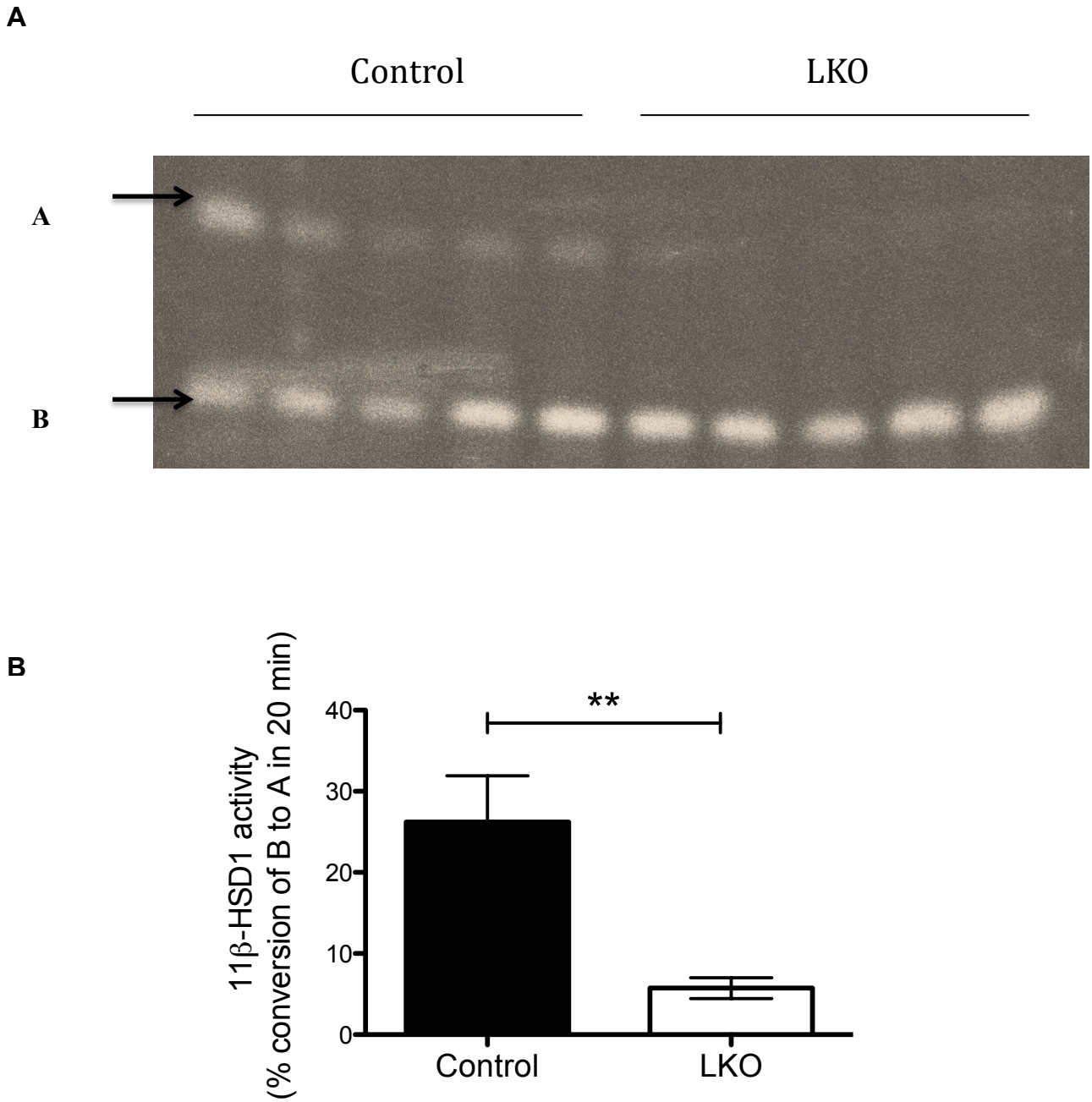


Figure 5.3: 11β-HSD1 enzyme activity is markedly reduced in livers of LKO mice.

(A) Image of the phosphorimager tritium image screen showing conversion of corticosterone (B) to 11-dehydrocorticosterone (A) in liver samples from male, chow-fed, 10 week old liver-specific 11β-HSD1 knockout (LKO) mice and their floxed *Hsd11b1* littermates (Control). Each lane represents one mouse, appropriately labeled. Steroids were separated by thin-layer chromatography in this experiment. **(B)** Density of bands in Figure 5.3A were quantified and plotted into a graph. Data are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=5/group; ** $p<0.001$.

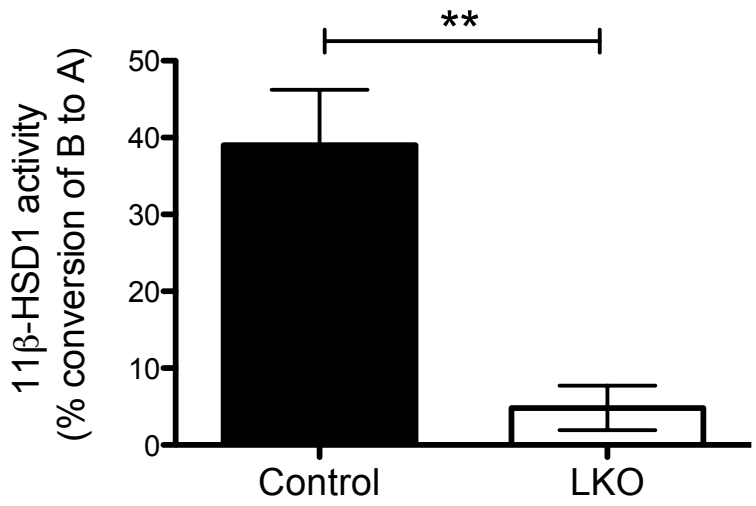


Figure 5.4: 11β-HSD1 enzyme activity is significantly reduced in livers of LKO mice.

A second 11β-HSD1 enzyme activity assay was carried out where steroids were separated by high-performance liquid chromatography (HPLC). Conversion of corticosterone (B) to 11-dehydrocorticosterone (A) was measured in liver samples from male, chow-fed, 10 week old liver-specific 11β-HSD1 knockout (LKO) mice and their floxed *Hsd11b1* littermates (Control). Data are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=6/group; ** $p<0.001$.

5.3.2. Similar 11β-HSD1 protein levels in epididymal fat, kidney and muscle of LKO and control mice

To confirm that 11β-HSD1 deletion is specific to the liver and had not occurred in other tissues (which normally express 11β-HSD1 in mice), western blot analyses were carried out to measure 11β-HSD1 protein levels in left sided epididymal fat, kidney and (quadriceps) muscle of chow-fed, male 10-week-old LKO and their littermate control floxed mice. As expected, there were no differences in 11β-HSD1 protein levels between LKO and control mice in any of the extrahepatic tissues examined: epididymal fat (Figure 5.5), kidney (Figure 5.6) and muscle (Figure 5.7).

It is interesting that western blot analysis in muscle showed two 11β-HSD1 protein bands, at approximately 50kDa and 51kDa (Figure 5.7), unlike the usual 34kDa band observed in liver, adipose tissue and kidney (Figures 5.2, 5.5 and 5.6). In previous 'test' western blot analyses, with fewer muscle samples, positive (liver from LOE mouse) and negative (liver from HSD1 KO mouse) controls were included to ensure that the antibody used recognises the targeted protein (11β-HSD1). Some studies have previously reported the presence of two (or more) 11β-HSD1 protein bands in western blot analyses (Maser *et al*., 2002), including a 50kDa band (Kannisto *et al*., 2004; Mariniello *et al*., 2006).

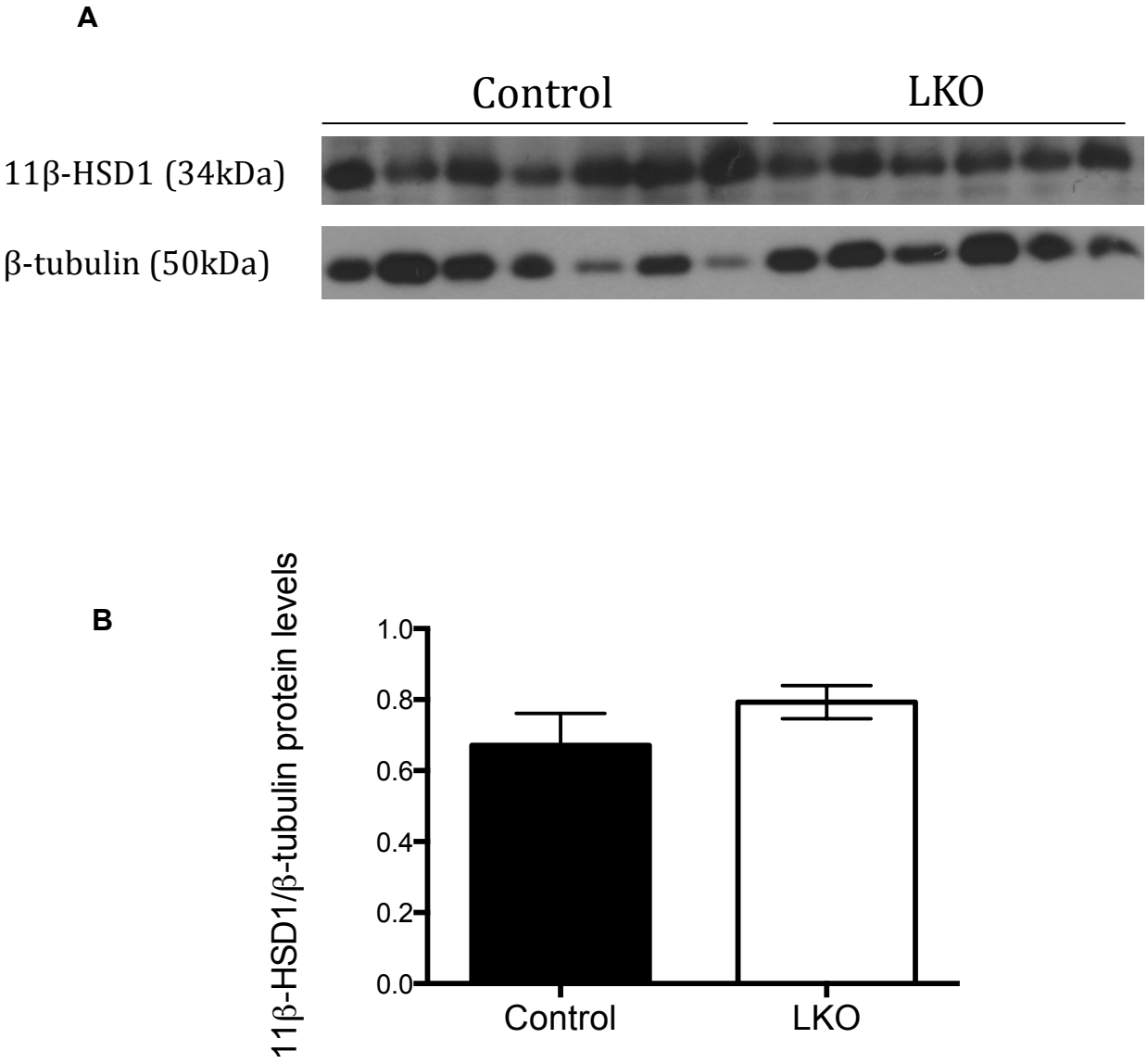


Figure 5.5: 11β-HSD1 protein is normally expressed in epididymal fat of LKO mice.

Western blotting was used to measure 11β-HSD1 protein levels in epididymal fat tissue from male, chow-fed, 10 week old floxed *Hsd11b1* (Control) and liver 11β-HSD1 knockout (LKO) mice. **(A)** Image of a representative western blot. Each lane contains 40μg protein from one mouse. β-tubulin was used as a loading control. **(B)** Density of bands in Figure 5.5A were quantified and plotted into a graph. Values are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=6-7/group.

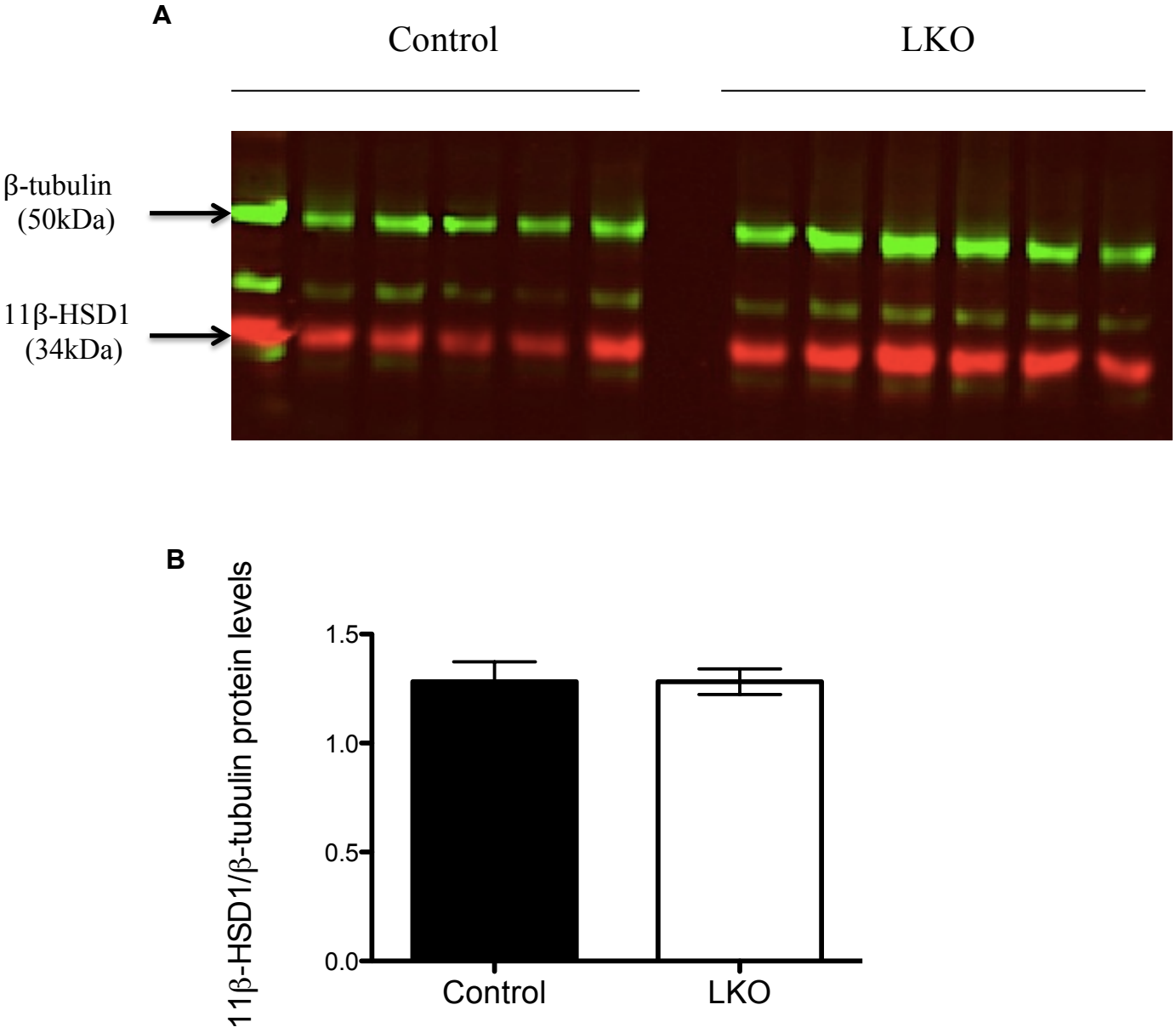


Figure 5.6: Similar levels of 11β-HSD1 protein are present in kidneys of LKO and control mice.

Western blotting was used to measure 11β-HSD1 protein levels (red) in kidneys of chow-fed, male, 10 week old *Hsd11b1* (Control) and liver 11β-HSD1 knockout (LKO) mice. **(A)** Image of a representative western blot. Each lane contains 40μg protein from one mouse. β-tubulin (in green) was used as a loading control. **(B)** Density of bands in Figure 5.6A were quantified and plotted into a graph. Values are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=6/group.

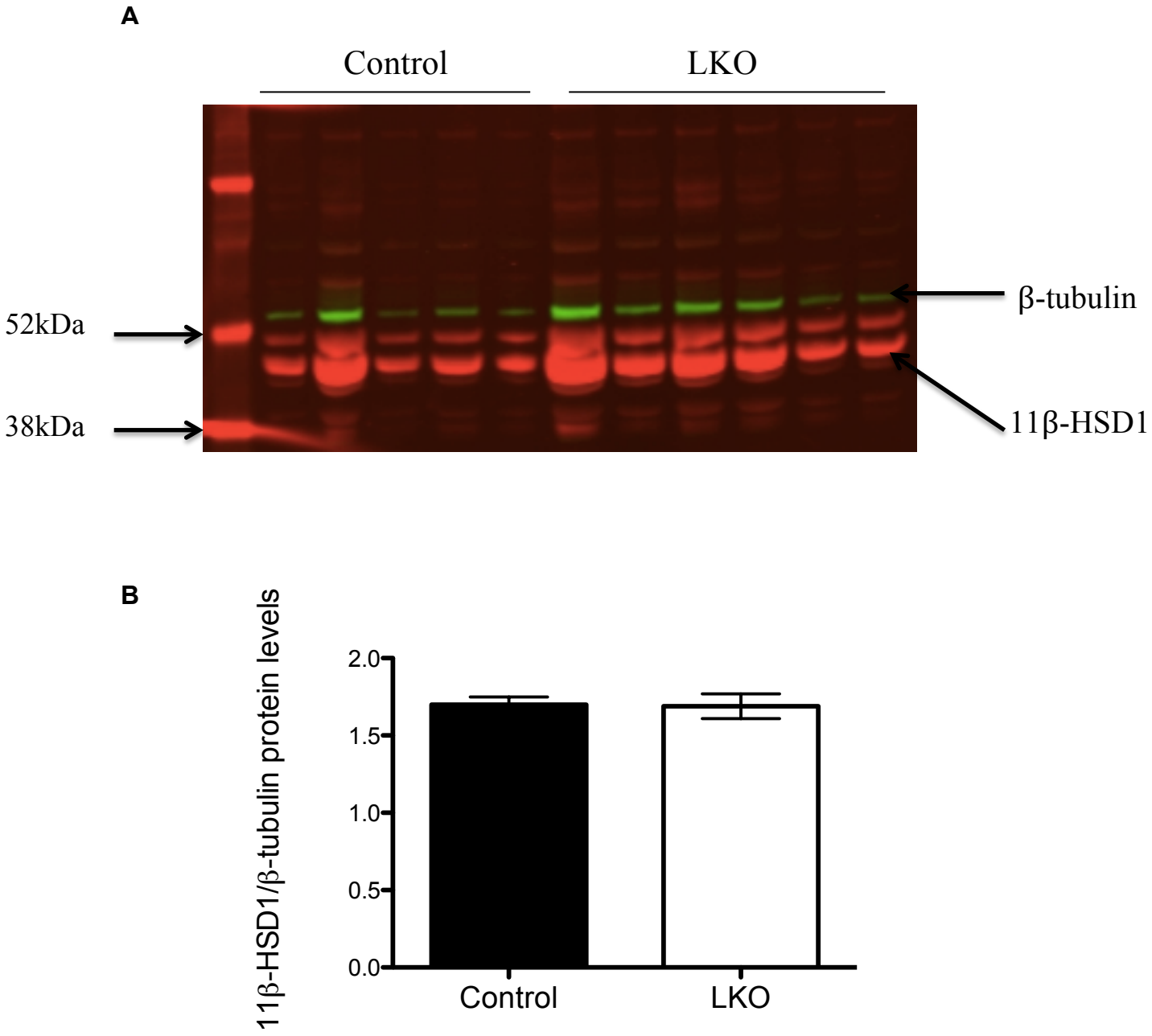


Figure 5.7: 11β-HSD1 protein is normally expressed in muscle in LKO mice.

Western blotting was used to examine 11β-HSD1 protein levels (red) in muscle of chow-fed, male, 10 week old floxed *Hsd11b1* (Control) and liver 11β-HSD1 knockout (LKO) mice. The left lane contains a protein ladder (some of the band sizes indicated). Next to the ladder are muscle protein samples from control and LKO mice, labelled appropriately. **(A)** Image of a representative western blot. Each lane contains 40μg protein from one mouse. β-tubulin (in green) was used as a loading control. **(B)** Density of bands in Figure 5.7A were quantified and plotted into a graph. Values are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=5-6/group.

5.3.3. Similar body weight and tissue weights between LKO and control mice

There was no difference in body weight between LKO and control mice (Figure 5.8). Tissue weight was recorded and normalised against body weight. As expected, there were no differences in tissue to body weight ratios between LKO and control mice in any of the tissues examined: liver (Figure 5.9A), epididymal fat (Figure 5.9B), kidney (Figure 5.9C), muscle (Figure 5.9D), or brain (Figure 5.9F).

Global 11β-HSD1 deficiency is known to stimulate the HPA axis. Previously, global 11β-HSD1 knockout mice showed increased adrenal weight compared to control mice (Kotelevtsev *et al*., 1997). Furthermore, hepatic GC regeneration has been implicated in the modulation of HPA axis sensitivity (Paterson *et al*., 2007), and consistent with this, Lavery *et al*. (2012) reported increased adrenal weight in their liver-specific 11β-HSD1 knockout mice (Lavery *et al*., 2012). However, in the current study, there were no differences in adrenal weight between LKO and control mice (Figure 5.9E).

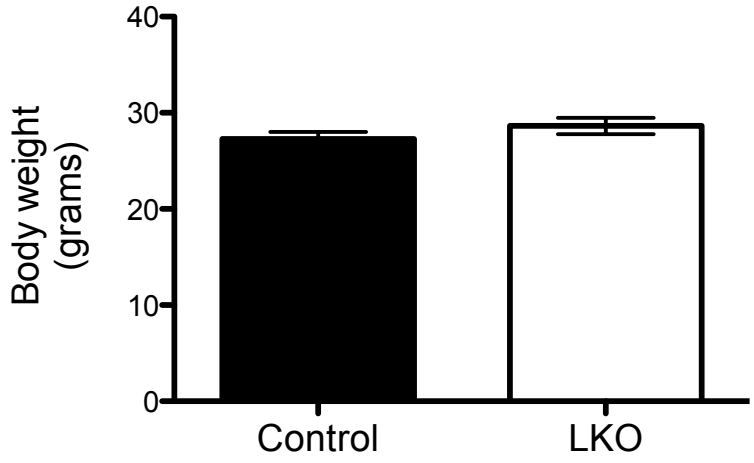


Figure 5.8: Similar body weight between LKO and control mice.

Chow-fed, 10 week old male, liver-specific 11β-HSD1 knockout (LKO) mice and their floxed *Hsd11b1* littermates (Control) had their body weights measured. Values are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=7-8/group.

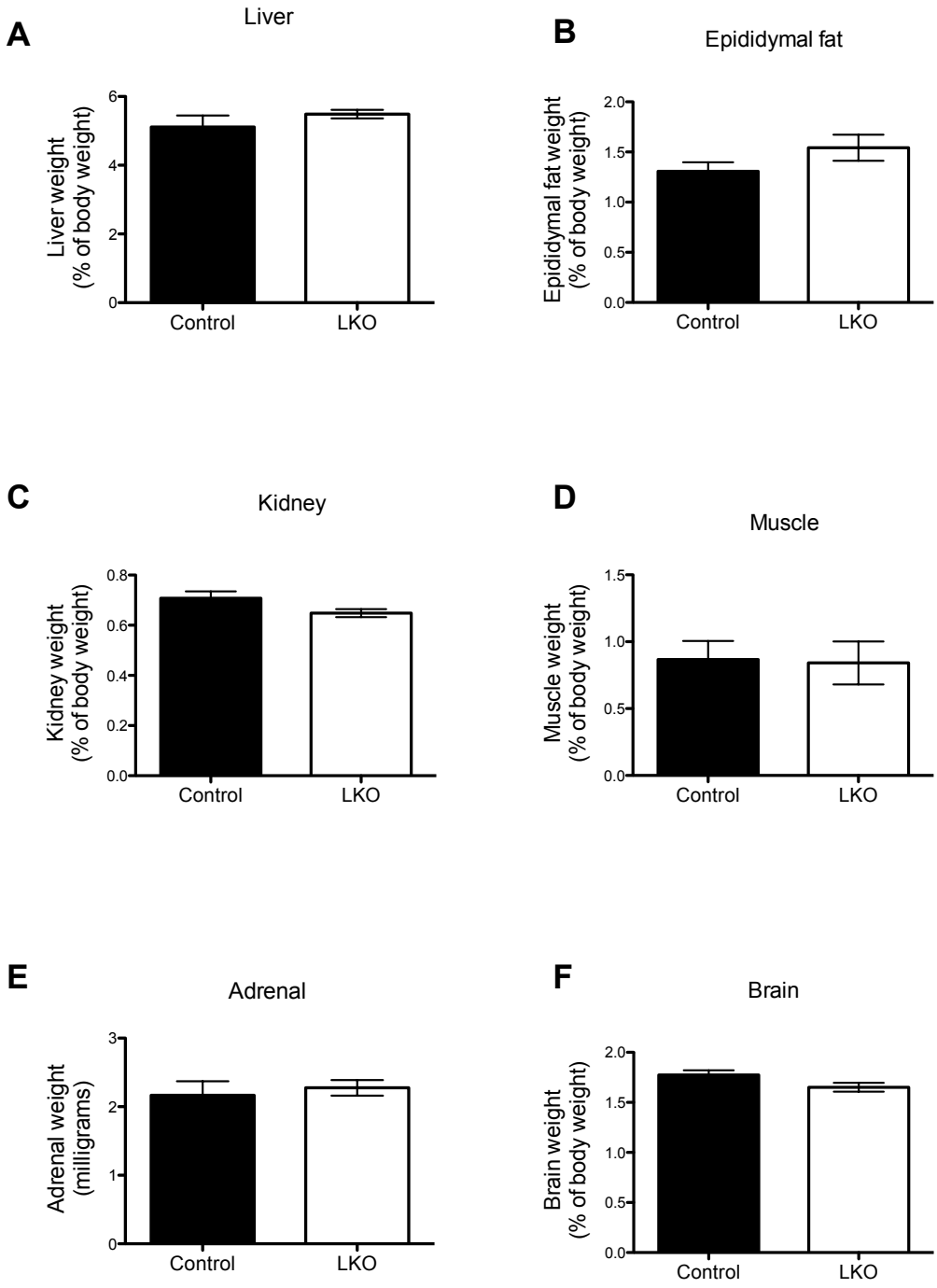


Figure 5.9: Similar tissue weights between LKO and control mice.

Tissues from male, chow-fed, 10 week old liver-specific 11β-HSD1 knockout (LKO) mice and their floxed *Hsd11b1* littermates (Control) were harvested and weighed. Weights of **(A)** liver, **(B)** epididymal fat, **(C)** kidney, **(D)** muscle, and **(F)** brain are expressed as percentage (%) of body weight. Adrenal weight **(E)** is shown in milligrams. Data are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=7-8/group.

5.3.4. Similar levels of hepatic *Srebp2*, *Hmgcr* and *Hmgcs* mRNA between LKO and control mice

Based on the original hypothesis, it was predicted that hepatic 11β-HSD1 deficiency would lead to decreased cholesterol synthesis (see 1.9). To test this, mRNAs encoding SREBP-2 and its target genes in the cholesterol biosynthetic pathway, such as HMGCR and HMGCS, were measured in livers from male, chow-fed 10 week old LKO mice and their floxed *Hsd11b1* littermate controls. Contradictory to predictions, there were no differences in the hepatic levels of *Srebp2* (Figure 5.10), *Hmgcr* (Figure 5.11A) or *Hmgcs* (Figure 5.11B) mRNA between genotypes, suggesting no change in cholesterol synthesis in LKO mice.

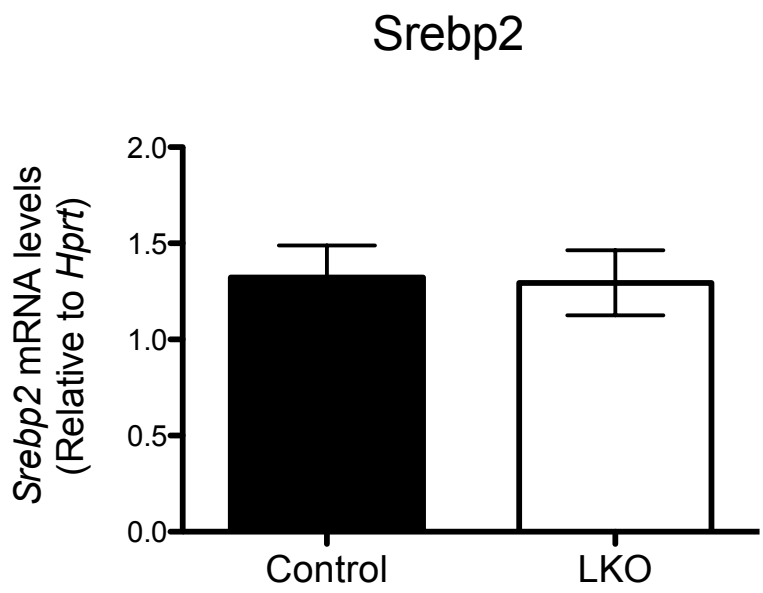


Figure 5.10: Similar hepatic *Srebp2* mRNA levels in LKO and control mice.

Total RNA was extracted from livers of male, chow-fed, 10 week old liver-specific 11β-HSD1 knockout (LKO) mice and floxed *Hsd11b1* littermates (Control). *Srebp2* mRNA levels were measured by qPCR and are expressed relative to *Hprt* mRNA levels, used as an internal control. Values are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=10-11/group.

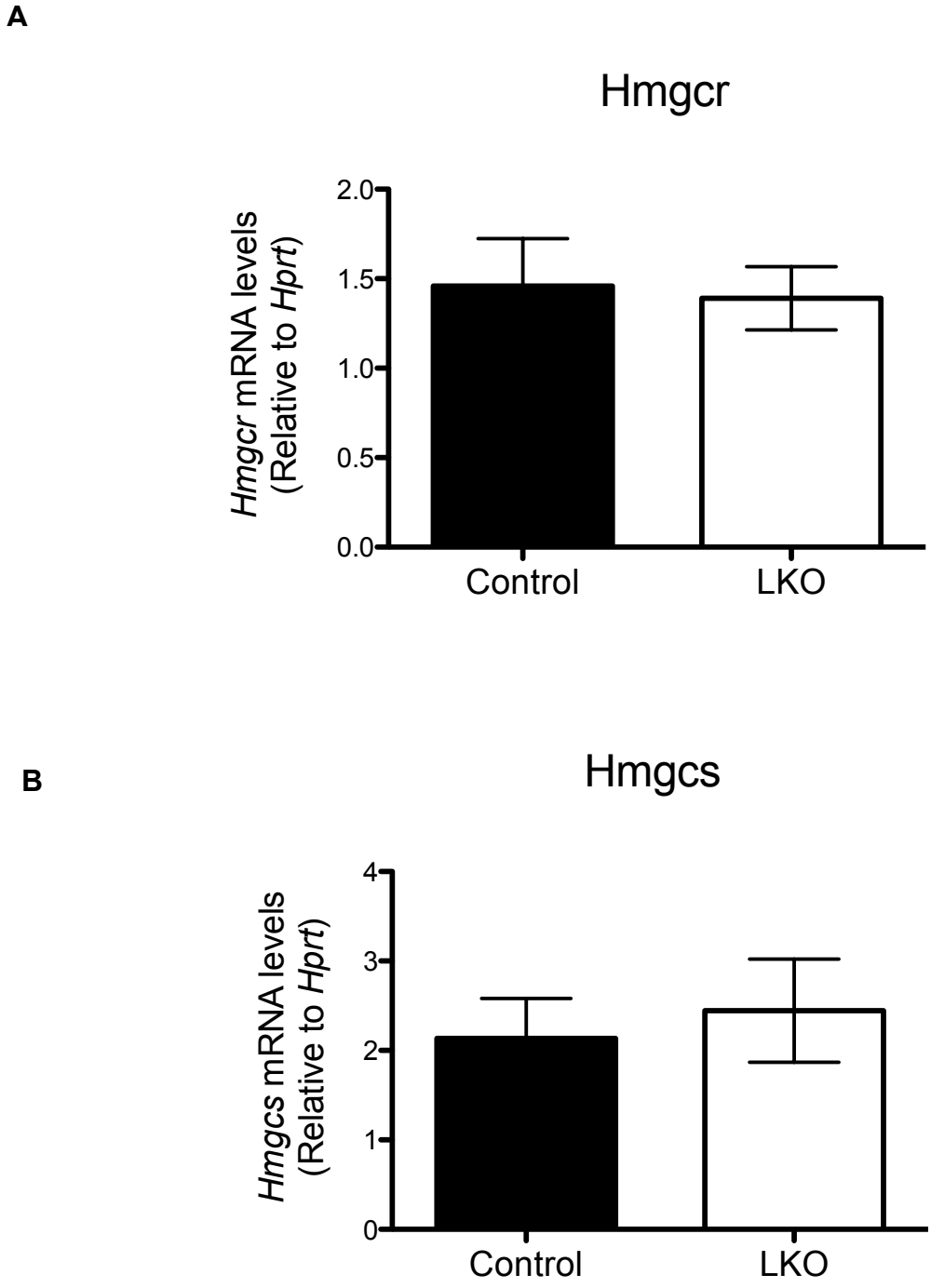


Figure 5.11: Levels of hepatic *Hmgcr* and *Hmgcs* mRNA do not differ between LKO and control mice.

Total RNA was extracted from liver of male chow-fed, 10 week old floxed *Hsd11b1* (Control) and liver-specific 11β-HSD1 knockout (LKO) mice. Messenger RNA levels were measured by qPCR. **(A)** *Hmgcr* and **(B)** *Hmgcs* mRNA levels are expressed relative to *Hprt* mRNA. Values are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=10-11/group.

5.3.5. Similar mRNA levels of hepatic *Lxrα*, as well as its target genes *Cyp7a1, Abcg5* and *Abcg8* between LKO and control mice

As previously discussed, LXRα plays a vital role in cholesterol homeostasis. Its activation is proportional to cellular cholesterol levels and leads to upregulation of genes that are involved in cholesterol catabolism (*Cyp7a1*) and cholesterol excretion (*Abcg5* and *Abcg8*) (Lehmann *et al*., 1997; Repa *et al*., 2002; Hong *et al*., 2012; see 1.5.5 and 3.3.4). LOE mice (both chow-fed and WD-fed) showed increased levels of hepatic *Lxrα* mRNA as well as elevated levels of hepatic *Abcg5* and *Abcg8* mRNA. To examine whether hepatic deficiency of 11β-HSD1 led to the opposite, *Lxrα, Abcg5* and *Abcg8* mRNAs were measured in livers of male, chow-fed, 10 week old LKO mice and their control littermates. Hepatic *Cyp7a1* mRNA levels were also measured in these mice to determine whether cholesterol catabolism via bile acid synthesis was altered with liver 11β-HSD1 deficiency.

Unlike LOE mice, LKO mice did not show any differences in the hepatic levels of *Lxrα* (Figure 5.12)*, Abcg5* (Figure 5.14A) or *Abcg8* (Figure 5.14B) mRNAs. Similar to findings in LOE mice, there was no difference in hepatic *Cyp7a1* mRNA expression between LKO and control mice (Figure 5.13).

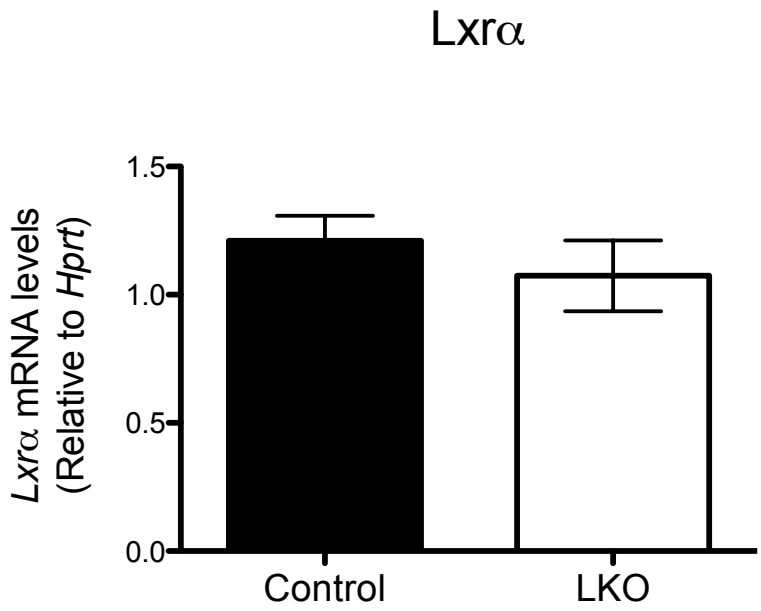


Figure 5.12: Similar hepatic *Lxrα* mRNA levels in LKO and control mice.

Total RNA was extracted from livers of male, chow-fed, 10 week old liver-specific 11β-HSD1 knockout (LKO) mice and their floxed *Hsd11b1* littermates (Control). *Lxrα* mRNA levels were measured by qPCR and are expressed relative to *Hprt* mRNA levels, used as an internal control. Data are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=10-11/group.

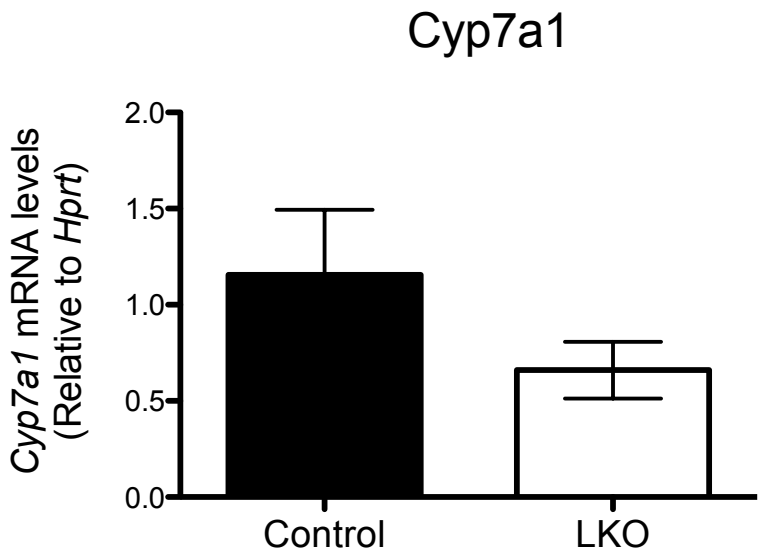


Figure 5.13: Similar hepatic *Cyp7a1* mRNA levels in LKO and control mice.

Total RNA was extracted from livers of male chow-fed, 10 week old liver-specific 11β-HSD1 knockout (LKO) mice and their floxed *Hsd11b1* littermates (Control). *Cyp7a1* mRNA levels were measured by qPCR and are expressed relative to *Hprt* mRNA levels, used as an internal control. Values are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=10-11/group.

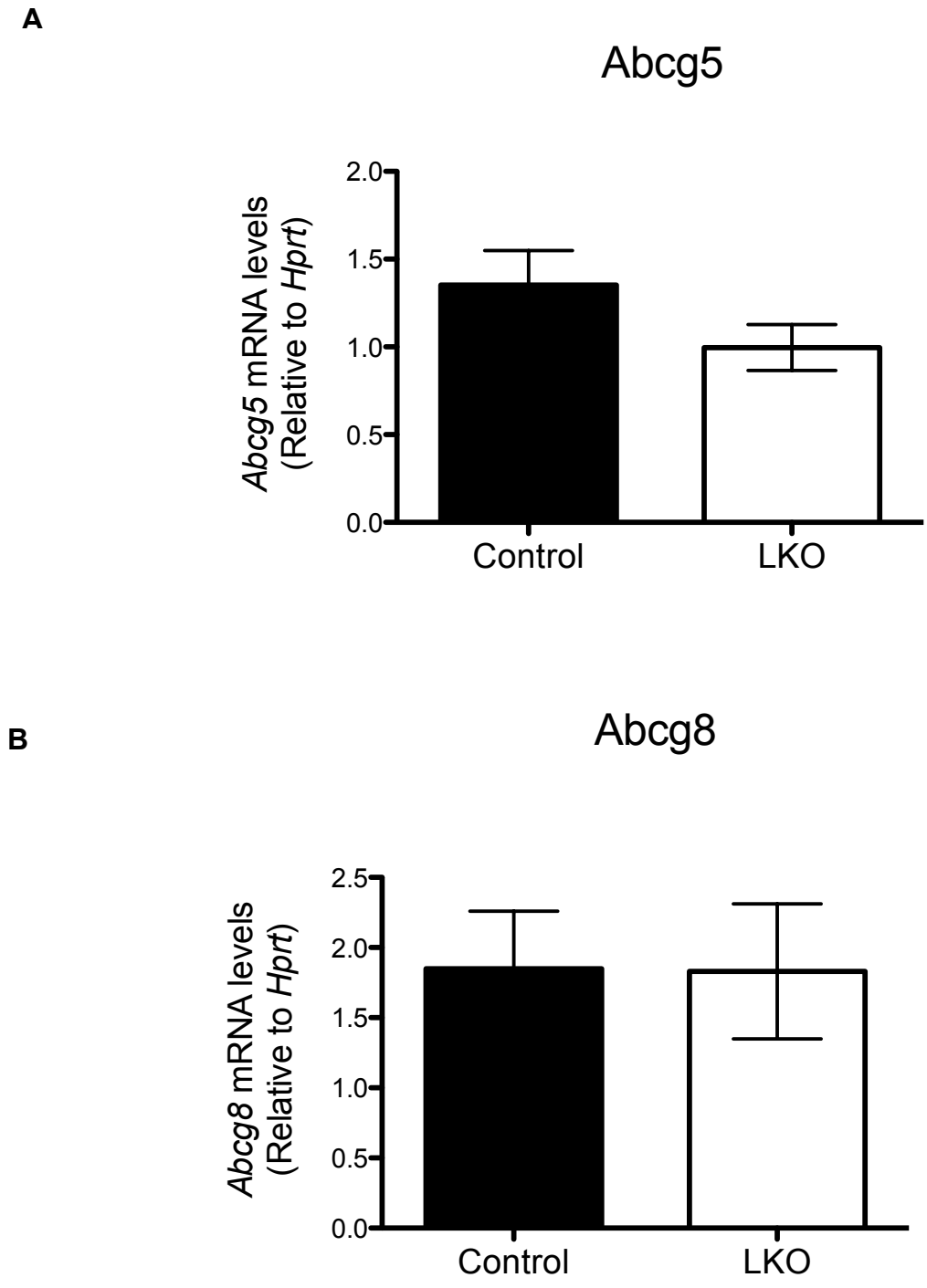


Figure 5.14: Levels of hepatic *Abcg5* and *Abcg8* mRNA do not differ between LKO and control mice.

Total RNA was extracted from livers of male, chow-fed, 10 week old floxed *Hsd11b1* (Control) and liver-specific 11β-HSD1 knockout (LKO) mice. Messenger RNA levels were measured by qPCR. **(A)** *Abcg5* and **(B)** *Abcg8* mRNA levels are expressed relative to *Hprt* mRNA. Data are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=10-11/group.

5.3.6. Similar levels of hepatic *ApoA1* mRNA between LKO and control mice

Apolipoprotein A1 mRNA, which encodes the major component of the HDL particle (Barter and Rye, 1996; see 4.3.11), has been previously shown to be elevated in livers of global 11β-HSD1 deficient mice, associated with increased serum ApoA1 levels and HDL cholesterol (Morton *et al*., 2001). To investigate whether hepatic 11β-HSD1 deficiency leads to increased ApoA1 expression, *ApoA1* mRNA levels were measured in livers of male, chow-fed, 10 week old LKO mice and their floxed *Hsd11b1* littermate controls. However, unlike global 11β-HSD1 knockout mice, there was no difference in the hepatic levels of *ApoA1* mRNA between LKO and control mice (Figure 5.15).

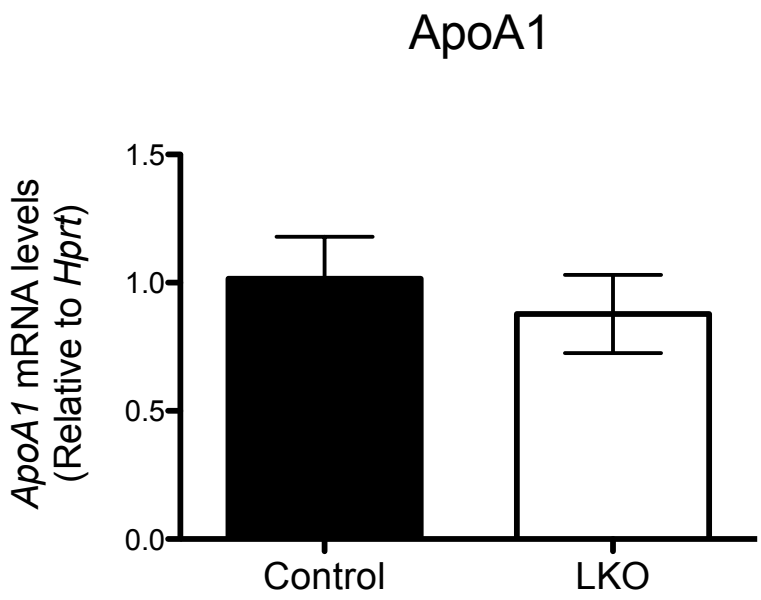


Figure 5.15: Similar hepatic *ApoA1* mRNA levels LKO and control mice.

Total RNA was extracted from livers of male, chow-fed, 10 week old liver-specific 11β-HSD1 knockout (LKO) mice and their floxed *Hsd11b1* littermates (Control). *ApoA1* mRNA levels were measured by qPCR and are expressed relative to *Hprt* mRNA levels, used as an internal control. Values are means ± SEM and were analysed by unpaired *t*-test (two-tailed); n=10-11/group.

5.4. Discussion

The line of liver-specific 11β-HSD1 deficient mice generated here display efficient Cre-mediated *Hsd11b1* recombination in liver. This is reflected in an almost 100% reduction in hepatic *Hsd11b1* mRNA and 11β-HSD1 protein levels in LKO mice compared with livers of their Cre^- littermate controls. Hepatic 11β-HSD1 enzyme activity was measured using two methods; TLC and HPLC. Results from the TLC experiment (n=5/group) revealed ~80% reduction in 11β-HSD1 enzyme activity in livers of LKO mice when compared to livers from control mice. While all LKO mice showed no hepatic 11β-HSD1 enzyme activity, there was a small amount of activity in the liver homogenate from one LKO mouse (Figure 5.3A). To confirm these results, hepatic 11β-HSD1 enzyme activity was also measured using HPLC. This experiment was carried out using the samples that were included in the TLC experiment together with liver samples from additional LKO mouse and control mouse belonging to a separate cohort (n=6/group). Results from this experiment also revealed no hepatic 11β-HSD1 enzyme activity in all the LKO samples except for the one that displayed a small amount of activity in the TLC experiment. It is possible that Cre excision is not 100% in some mice but it would be worth re-genotyping this particular sample to confirm its identity.

There were no differences in 11β-HSD1 protein levels in epididymal fat, muscle or kidney between LKO and control mice. Along with the above-mentioned data, this suggests that LKO mice have a liver-specific knockout of 11β-HSD1.

LKO mice were generated primarily to examine the effect of hepatic 11β-HSD1 knockdown on metabolic parameters including body weight, tissue weights and expression of genes involved in cholesterol homeostasis. There was no effect of hepatic 11β-HSD1 deficiency upon body weight, liver weight or adiposity. Unexpectedly, no difference was observed in the adrenal weights between LKO and control mice. Global knockdown of 11β-HSD1 is known to stimulate the HPA axis. In a previous study, global 11β-HSD1 deficient mice displayed increased adrenal weight compared to control mice (Kotelevtsev *et al*., 1997). This HPA axis

dysfunction was rescued when global 11β-HSD1 deficient mice were crossed with liver-specific 11β-HSD1 overexpressor (LOE) mice (Paterson *et al*., 2007), suggesting that hepatic glucocorticoid regeneration regulates HPA axis sensitivity. When generation of mice with liver-specific deletion of *Hsd11b1* commenced in our lab, there were no reports on a liver-specific 11β-HSD1 knockout mouse model. However, recently, investigators from other labs have published their work on liver-specific 11β-HSD1 deficiency. A study by Lavery *et al*. (2012) that generated liver-specific 11β-HSD1 deficient mice (on a mixed C57BL/6/129SvJ background) reported significantly increased adrenal weight in their liver 11β-HSD1 knockout mice (Lavery *et al*., 2012). Although 11β-HSD1 deficiency on a C57BL/6 background results in a less dramatic adrenal weight increase compared to 11β-HSD1 deficiency on a 129 background (Carter *et al*., 2009), both C57BL/6 and 129SvJ strains reveal adrenal hypertrophy with disruption of 11β-HSD1 (Kotelevtsev *et al*., 1997; Carter *et al*., 2009) and, therefore, it is unlikely that background is the reason behind the lack of effect on adrenal gland weight in my line of LKO mice. The in-house *Hsd11b1*$^{flox/flox}$ mice that were used as controls in the present study may display some reduction in 11β-HSD1 activity and could be a possible reason for the unaltered adrenal weight in LKO mice.

Although predicted to decrease in LKO mice, no difference was observed in the hepatic expression of *Srebp2*, *Hmgcr* and *Hmgcs* mRNA levels between LKO and control mice, suggesting that cholesterol synthesis is not altered with the removal of hepatic 11β-HSD1. However, with hindsight, this is probably not surprising, as data from LOE mice do not reveal increased hepatic *Hmgcr* and *Hmgcs* mRNA expression as hypothesised.

As LOE mice displayed elevated hepatic *Lxrα* mRNA levels, removal of hepatic 11β-HSD1 was predicted to result in decreased *Lxrα* mRNA expression. However, there was no difference in hepatic *Lxrα* mRNA levels between LKO mice and their Cre^{-} littermates. Furthermore, no differences were observed in hepatic *Abcg5* and *Abcg8* mRNA levels between genotypes. This is inconsistent with a recent study that reported reduced hepatic *Abcg5* and *Abcg8* mRNA levels following antisense oligonucleotide knock down of 11β-HSD1 in livers of C57Bl/6 mice (Li *et al*., 2011).

However, the study carried out by Li *et al*. (2011) used a Western-type diet with a high content of fat and cholesterol as opposed to the chow diet used in the present study. Moreover, it is also worth noting that the antisense oligonucleotide did not simply knock down 11β-HSD1 in liver but also down-regulated 11β-HSD1 in most adipose tissue depots (Li *et al*., 2011), in effect making their model a knockout for adipose tissue as well as liver 11β-HSD1.

In conclusion, liver-specific 11β-HSD1 deficiency is achieved in LKO mice. There is, however, no evidence that hepatic 11β-HSD1 deficiency alters cholesterol homeostasis, at least with chow diet feeding. This is demonstrated by normal levels of mRNAs encoding hepatic cholesterol biosynthetic enzymes as well as those encoding enzymes/transporters for cholesterol catabolism/excretion in LKO mice. These data also indicate that the suggested role for hepatic 11β-HSD1 in hepatobiliary cholesterol excretion is specific to liver 11β-HSD1 overexpression.

Chapter 6. Effect of Hepatic 11β-HSD1 Deficiency combined with High Fat Feeding on Cholesterol Homeostasis and other Metabolic Parameters

6.1. Introduction

Although results in Chapters 3-5 do not indicate a role for hepatic 11β-HSD1 in cholesterol biosynthesis, levels of hepatic *Abcg5* and *Abcg8* mRNA (LXRα targets) were significantly increased with liver-specific 11β-HSD1 overexpression, both on standard chow diet as well as Western-style diet. These data suggest that liver 11β-HSD1 overexpression promotes hepatobiliary cholesterol efflux, consistent with a role for hepatic 11β-HSD1 in cholesterol homeostasis. Although no differences were observed in hepatic *Lxrα*, *Abcg5* and *Abc8* mRNA levels between chow-fed LKO mice and controls (Chapter 5), it is possible that diet is an influential factor, or that 11β-HSD1 action in another tissue (e.g. adipose, macrophages) compensated for hepatocytes in this model.

11β-HSD1 is most abundantly expressed in the liver, and data from the original study on global 11β-HSD1 knockout mice highlighted liver as an important site for 11β-HSD1 effects on glucose homeostasis and type 2 diabetes (Chapter 1, see 1.4.1). Several rodent studies have demonstrated the potential of selective 11β-HSD1 inhibitors to treat the components of metabolic syndrome as well as atherosclerosis (see 3.1), and some human clinical data have shown 11β-HSD1 inhibition to reduce weight and blood glucose levels in patients with type 2 diabetes poorly controlled by metformin therapy (Rosenstock *et al*., 2010). However, it is important to understand the specific sites and mechanisms of action involved, to improve therapy for type 2 diabetes, dyslipidaemia, obesity and atherosclerosis.

To examine the *in vivo* effects of hepatic 11β-HSD1 deficiency and dietary risk factors upon hepatic cholesterol homeostasis as well as other metabolic parameters, a HF diet experiment was carried out with liver-specific 11β-HSD1 deficient (LKO) mice. Previous studies from our lab revealed that 11β-HSD1 knockout mice resist hyperglycaemia on HF diet, display increased insulin sensitivity in liver and adipose tissue, and have decreased visceral fat upon HF feeding (see 1.4.1). To investigate if this was due to liver 11β-HSD1, LKO mice were fed HF diet in this experiment (see 6.2). There are no published data from our lab on WD-fed 11β-HSD1 knockout mice,

except on *Apoe*$^{-/-}$ background, and because LKO mice were not on the *Apoe*$^{-/-}$ background, a Western diet experiment was not carried out on them.

Male, 8-10 week old LKO mice, floxed *Hsd11b1* mice (Control), complete global 11β-HSD1 deficient (Del), and 'old' hypomorphic 11β-HSD1 knockout (HSD1 KO) mice were fed HF diet for 14 weeks. Body weights were measured weekly. A glucose tolerance test (GTT) was carried out in week 13 before the mice were culled for tissue collection in week 14. This study was part of a larger experiment that also included other in-house generated tissue-specific 11β-HSD1 knockout mice (adipose-specific and macrophage-specific knockout models) on the HF diet.

The 'old' line of 11β-HSD1 knockout mice (referred to as HSD1 KO) is hypomorphic, showing partial 11β-HSD1 activity in some tissues, notably lung, where most of the 11β-HSD1 is expressed in fibroblasts (Coutinho, 2009; Yang, 2010). Recently, mice with complete loss of *Hsd11b1* were generated in our lab by germline deletion of "floxed" *Hsd11b1*. The complete 11β-HSD1 knockout line (referred to as Del here) carry the identical deletion of exon 3 of *Hsd11b1* to LKO mice, and were included in this study as positive controls.

6.2. Hypothesis and Aims

At the stage when this PhD project started, the following hypothesis was proposed.

Hypothesis: Liver 11β-HSD1 deficiency, combined with HF feeding, will reduce hepatic cholesterol synthesis via reduced hepatic expression of genes encoding cholesterol biosynthetic enzymes.

In the light of data from Chapters 3, 4 and 5, with hindsight, it now appears that this might not be the case. Nevertheless, in keeping with investigation of the role of hepatic 11β-HSD1 in cholesterol homeostasis (evidence for increased hepatobiliary cholesterol secretion with hepatic 11β-HSD1 overexpression; Chapters 3 and 4), the following work was started at the same time as that carried out in Chapter 5. The original aims are listed below.

In addition, this HF diet experiment was carried out with various tissue-specific (liver, adipose and macrophage) 11β-HSD1 knockout lines to examine the locus for the beneficial metabolic effects of global 11β-HSD1 deficiency. Given that liver seems to be the target tissue for anti-diabetic effects of 11β-HSD1 deficiency as well as one of the major sites for lipid and cholesterol metabolism, it was hypothesised that liver-specific 11β-HSD1 deficiency will partly or fully account for the protective metabolic effects of global 11β-HSD1 deficiency.

Aims

1) To investigate the effects of liver 11β-HSD1 deficiency combined with HF feeding on metabolic parameters such as body weight gain, liver and adipose tissue weights, and glucose homeostasis.

2) To determine if liver-specific 11β-HSD1 deficiency, upon HF feeding, leads to altered hepatic expression of mRNAs encoding SREBP-2 and its target cholesterol biosynthetic enzymes.

3) To test whether hepatic 11β-HSD1 deficiency combined with HF feeding leads to decreased hepatic levels of mRNAs encoding LXRα and its downstream targets involved in cholesterol efflux and cholesterol clearance.

6.3. Results

6.3.1. Decreased adipose tissue weight in HF-fed LKO mice compared to HF-fed controls

LKO mice showed no difference in body weight gain compared with control *Hsd11b1* floxed mice during the entire 14-week duration of the HF diet (Figure 6.1). In contrast, Del and HSD1 KO mice showed reduced weight gain compared to control mice towards the end of this experimental study; in Del mice this was significant from week 11 until the final week (14) of diet, and for HSD1 KO mice, at weeks 13 and 14 of the study (Figure 6.1).

Tissue weights were recorded and normalised against body weights. There were no differences in tissue to body weight ratios between genotypes in either liver (Figure 6.2A) or adrenal gland (Figure 6.2B). However, intriguingly, HF-fed LKO mice showed significantly reduced weights for subcutaneous fat (Figure 6.2D) as well as for epididymal fat (Figure 6.2E) when compared with HF-fed control mice, and mesenteric fat showed a similar trend though this was not significant (Figure 6.2C).

Previously, HF-fed HSD1 KO mice have shown a general reduction in fat mass gain (Morton *et al*., 2004; Wamil *et al*., 2011). Furthermore, reduced subcutaneous adipocyte hypertrophy was observed in HF-fed HSD1 KO mice compared to HF-fed C57BL/6 control mice (Wamil *et al*., 2011). To investigate if decreased adiposity in LKO mice was due to reduced hypertrophy in their adipose tissue, adipocyte sizes were measured in picrosirius red stained subcutaneous fat samples. There were no differences in the size of subcutaneous adipocytes between LKO and control mice (or HSD1 KO mice) (Figure 6.3), suggesting that the decrease in adipose tissue weight observed in LKO mice is not due to less hypertrophy in subcutaneous fat.

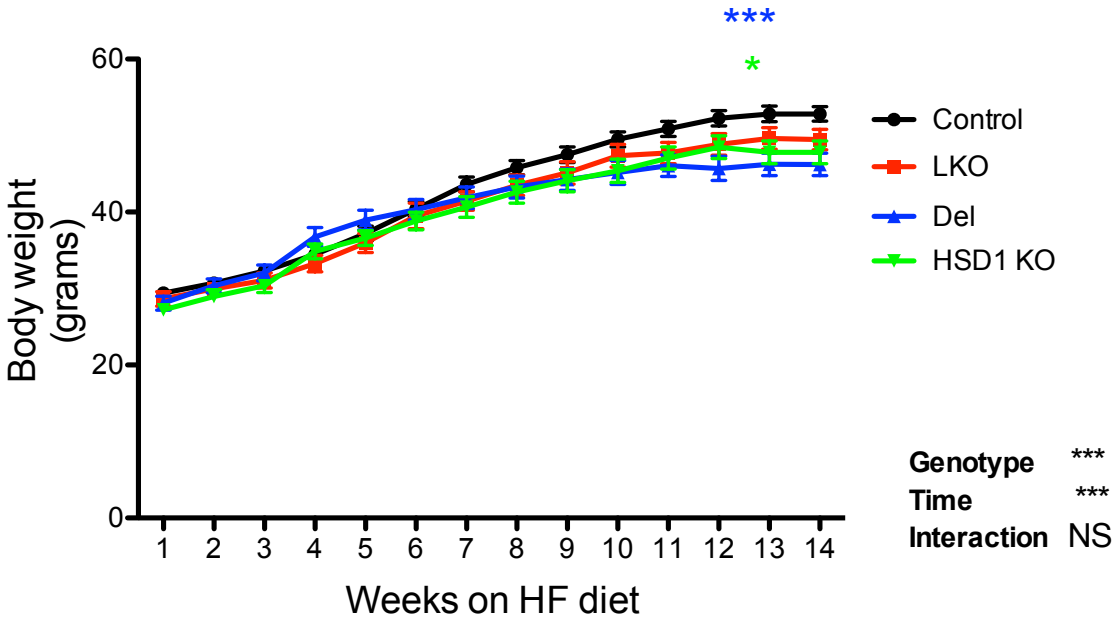


Figure 6.1: LKO mice do not show the reduction in body weight gain seen in Del and HSD1 KO mice with high fat diet feeding.

Body weight was monitored weekly in experimental mice during 14-week high fat diet feeding (from 8-10 weeks of age to 22-24 weeks of age). Red squares show liver-specific 11β-HSD1 knockout (LKO) mice, blue triangles show complete global 11β-HSD1 deficient (Del) mice, green triangles show 'old' hypomorphic 11β-HSD1 knockout (HSD1 KO) mice, and black circles show floxed *Hsd11b1* (Control) mice. Data are means ± SEM and were analysed by repeated measures two-way ANOVA with Bonferroni post test; n=7-16/group. Del mice had significantly reduced weight gain compared to control mice from week 11 to the end of the study (week 14; *** $p<0.001$). HSD1 KO mice had significantly decreased weight gain rate compared to control mice at weeks 13 and 14 of the study (* $p<0.05$).

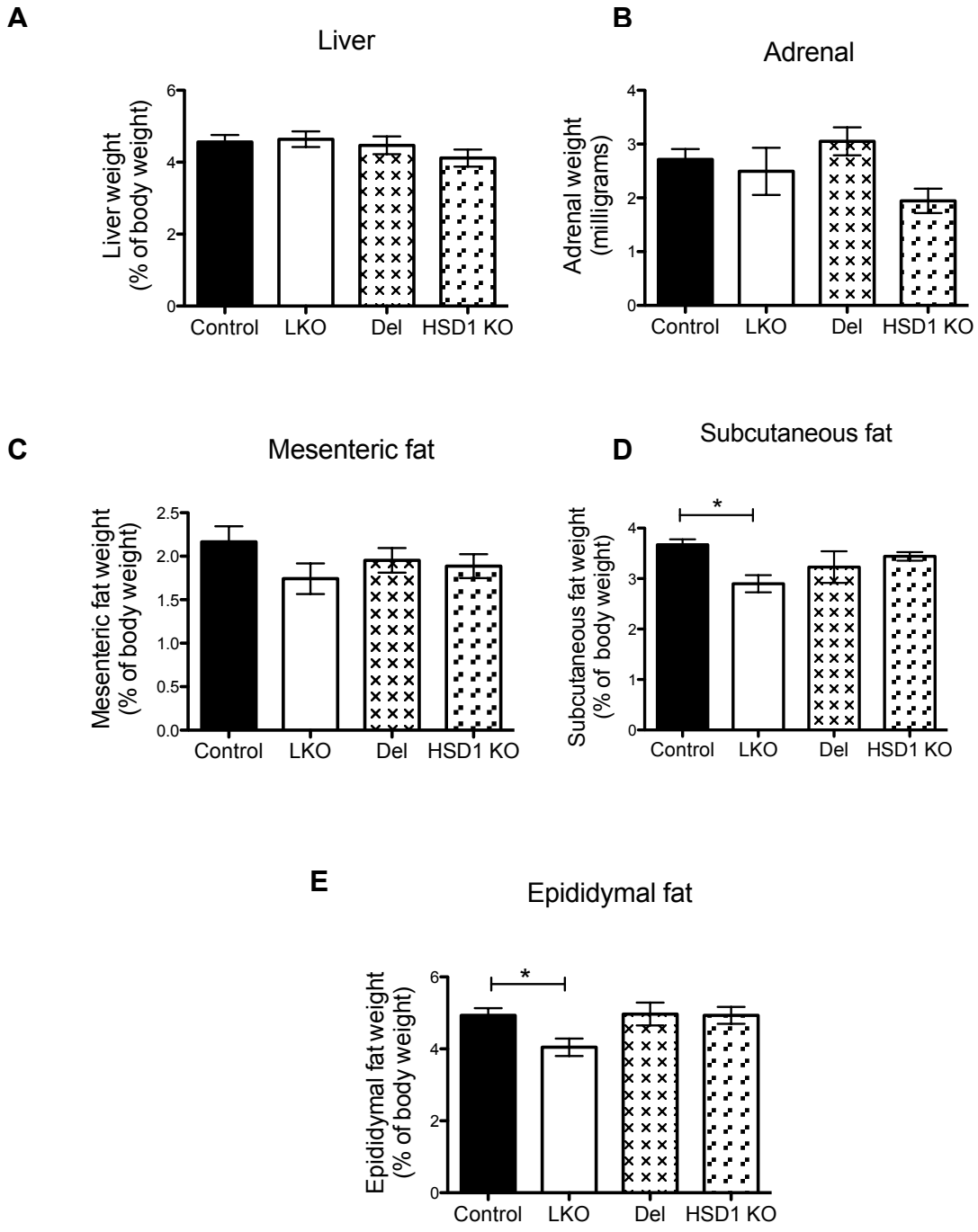


Figure 6.2: Reduced adipose tissue weight in LKO mice compared to controls.

At the end of the 14-week high fat diet experiment, tissues were harvested from the mice- liver-specific 11β-HSD1 knockout (LKO), floxed *Hsd11b1* (Control), 'new' complete global 11β-HSD1 deficient (Del), and 'old' hypomorphic 11β-HSD1 knockout (HSD1 KO). Weights of **(A)** liver, **(B)** adrenal, **(C)** mesenteric fat, **(D)** subcutaneous fat and **(E)** epididymal fat of the mice are shown. All tissue weights (except adrenal) are expressed as % of body weight. Data are means ± SEM and were analysed by one-way ANOVA with Tukey's multiple comparisons test; n=7-14/group; * $p<0.05$.

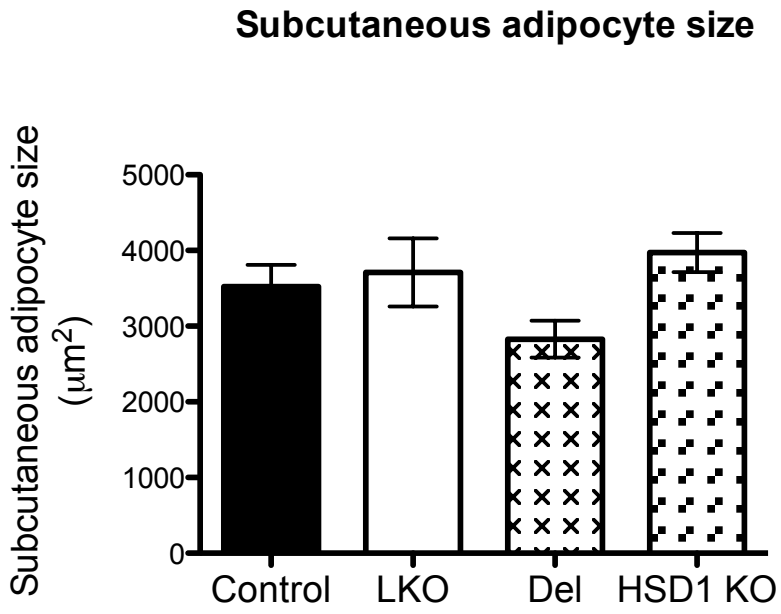


Figure 6.3: Similar adipocyte size in subcutaneous fat of LKO mice compared to subcutaneous fat of control mice.

Picrosirius red staining was performed on sections of subcutaneous adipose tissue samples collected from liver-specific 11β-HSD1 knockout (LKO) mice, complete global 11β-HSD1 deficient (Del) mice, 'old' hypomorphic 11β-HSD1 knockout (HSD1 KO) and floxed *Hsd11b1* (Control) mice that were fed high fat diet for 14 weeks. Adipocyte sizes in the stained tissue sections were measured using ImageJ. Data are means ± SEM and were analysed by one-way ANOVA with Tukey's multiple comparisons test; n=4-6/group. Quantification was performed by Oliver Brown, an undergraduate student.

6.3.2. Opposing effects of null and hypomorphic alleles of *Hsd11b1* on adipose tissue collagen content but no effect of liver 11β-HSD1 deletion on adipose tissue collagen

Michailidou *et al*. (2012) previously reported that HF-fed HSD1 KO mice exhibit reduced collagen deposition, a key feature of fibrosis (Iredale, 2007), in both subcutaneous and mesenteric fat depots compared to control mice (Michailidou *et al*., 2012).

Given the impact of liver-specific 11β-HSD1 deficiency on adipose tissue weight and to test the involvement of hepatic 11β-HSD1 in adipose tissue remodelling, collagen deposition was measured in picrosirius red stained subcutaneous fat sections. Quantification of the staining was carried out by Mr. Oliver Brown, an undergraduate project student; this was performed blinded to the group, and the work was checked by me.

There was a strong trend for reduced collagen deposition in subcunatoeus fat of HF-fed HSD1 KO mice compared to HF-fed controls though this did not achieve significance (Figure 6.4B). This may be because of low number of samples (therefore, a type II error). Nevertheless, there was a significant difference in collagen deposition between subcunatoeus fat depots of HF-fed Del and HSD1 KO mice; increased collagen deposition was observed in subcutaneous fat of HF-fed Del mice compared to HSD1 KO subcutaneous adipose tissue (Figure 6.4B), suggesting increased fibrosis in subcutaneous fat of HF-fed Del mice (at least) when compared with subcutaneous fat of HF-fed HSD1 KO mice. It is also worth noting that although Del mice appear to exhibit increased subcutaneous adipose collagen deposition compared to control mice (Figure 6.4B), this did not reach significance. These preliminary findings merit replication.

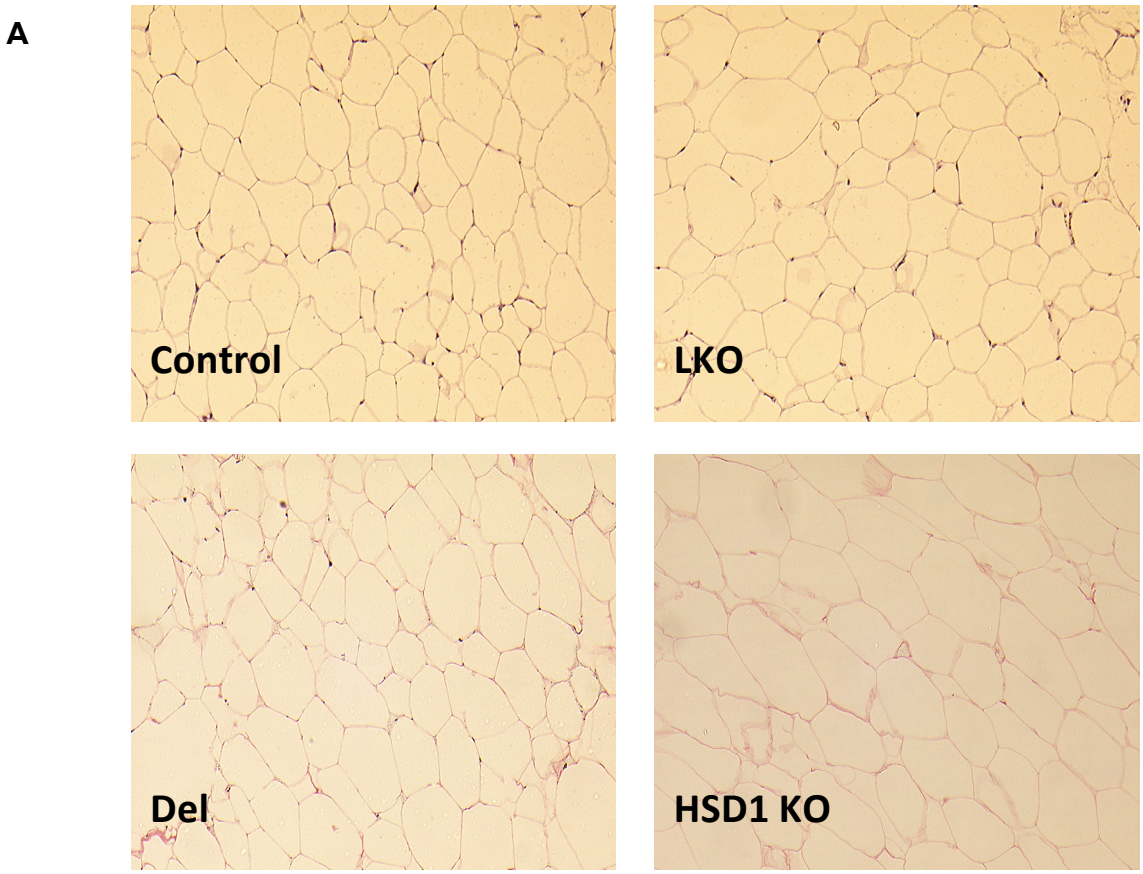


Figure 6.4: Increased collagen content in subcutaneous fat of Del mice compared to HSD1 KO subcutaneous fat.

(A) Representative images of picrosirius red stained sections of subcutaneous adipose tissue samples from liver-specific 11β-HSD1 knockout (LKO), floxed *Hsd11b1* (Control), complete global 11β-HSD1 deficient (Del) and 'old' hypomorphic 11β-HSD1 knockout (HSD1 KO) mice, which were high fat-fed for 14 weeks from 8-10 weeks of age. **(B)** Collagen staining in the tissue sections was quantified using ImageJ. Data are means ± SEM and were analysed by one-way ANOVA with Tukey's multiple comparisons test; n=4-8/group. Quantification of collagen staining was performed by Oliver Brown, an undergraduate student.

6.3.3. Increased circulating glucose levels during GTT in LKO mice compared to HSD1 KO mice

Previous studies have shown that global HSD1 KO mice display improved glucose tolerance when fed HF diet and an attenuated gluconeogenic response to fasting compared to wild-type mice (Kotelevtsev *et al*., 1997; Morton *et al*., 2004; see 1.4.1). Also, pharmacological inhibition of 11β-HSD1 improves glycaemia and increases hepatic insulin sensitivity in both humans (Walker *et al*., 1995; Andrews *et al*., 2003; Rosenstock *et al*., 2010) and obese, insulin-resistant rodents (Alberts *et al*., 2002; Livingstone and Walker, 2003; see 1.4.1).

Intraperitoneal glucose tolerance tests (GTT) were performed to assess whether liver-specific 11β-HSD1 deficiency is protective against HF diet-induced hyperglycaemia. Blood glucose levels during the GTT were measured immediately using a glucose meter as well as in stored plasma samples with a glucose assay. The limited detection range of the glucose meter resulted in several "HI" readings without a numerical value. Because many data points exceeded the upper limit of the assay and were unusable, no statistical test was performed on the GTT glucose levels measured by the glucose meter and the data are not shown. Plasma glucose and insulin measurements were carried out by Dr. Sophie Turban, and appropriate statistical tests were performed to analyse these data.

At 60 min after glucose injection, LKO mice showed a significant increase in glucose level compared to HSD1 KO mice at the same time point (Figure 6.5A). By comparing area under the curve, there was a trend for reduced glucose levels during GTT in HSD1 KO mice compared to control mice (Figure 6.5B) though it did not achieve significance. This might be because the sample size of HSD1 KO mice in this experiment is smaller than those for other genotypes, especially the controls. Nonetheless, in comparison with HSD1 KO mice, LKO mice displayed significantly elevated glucose levels during GTT (Figure 6.5B).

There were no differences in fasting glucose levels between LKO and control mice (Figure 6.6). In contrast, both HSD1 KO and Del mice showed significant reduction in fasting glucose levels compared to control mice (Figure 6.6). It is worth noting that

the fasting glucose levels observed in this study are quite high (Figure 6.6) (usual levels ranging between 4-7mM in mice (and humans)), suggesting these mice are highly insulin resistant.

Plasma insulin levels during GTT showed no differences between genotypes, using two-way repeated measures ANOVA (Figure 6.7A) or by comparing area under the curve (Figure 6.7B).

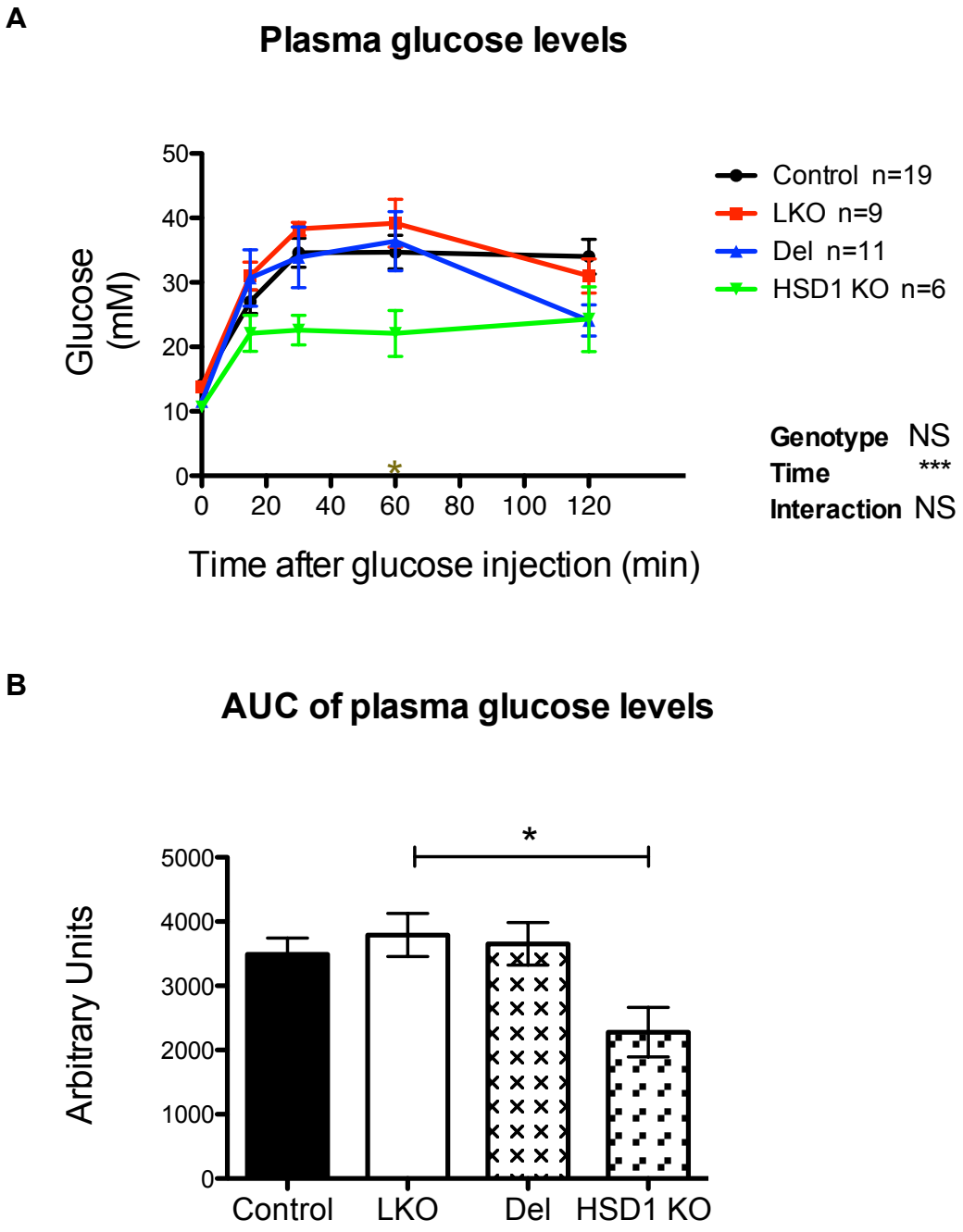


Figure 6.5: Increased glucose levels during GTT in LKO mice compared to HSD1 KO mice.

Male, 8-10 week old liver-specific 11β-HSD1 knockout (LKO; red squares), *Hsd11b1* floxed (Control; black circles), complete global 11β-HSD1 deficient (Del; blue triangles), and 'old' hypomorphic 11β-HSD1 knockout (HSD1 KO; green triangles) mice were subject to 14-week high fat diet, with glucose tolerance testing (GTT) carried out in week 13 of the dietary experiment. Circulating glucose levels were measured by **(A)** a glucose assay in the plasma. Data were analysed using two-way repeated measures ANOVA with Bonferroni post test; n=6-19/group. **(B)** Area under curve (AUC) of plasma glucose levels during GTT between genotypes. Data were analysed using one-way ANOVA with Tukey's multiple comparisons test; n=6-19/group.

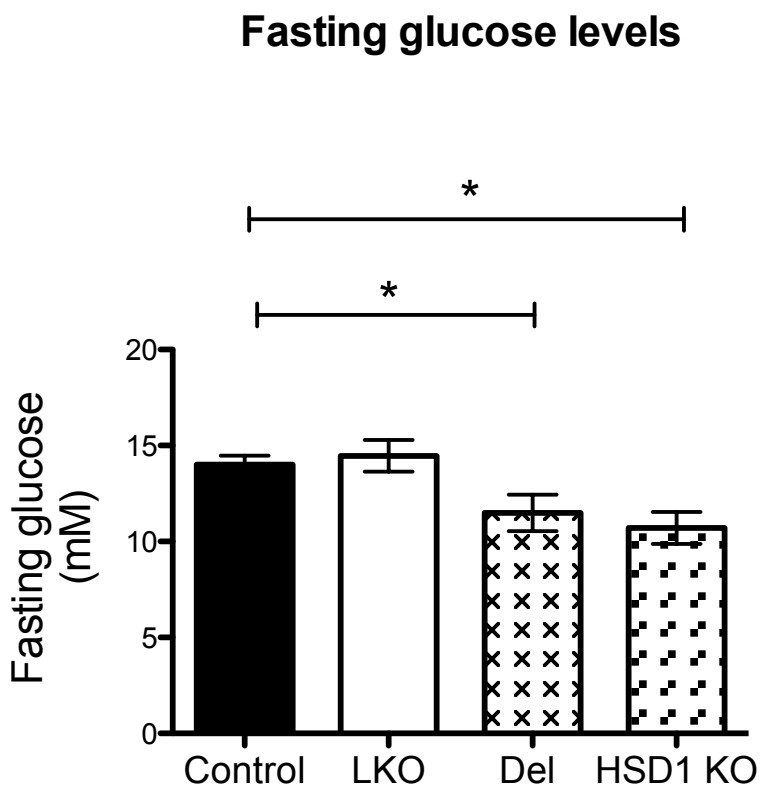


Figure 6.6: Reduced fasting glucose levels in Del and HSD1 KO mice compared to control mice.

During the experiment described in Figure 6.5 fasting glucose levels were measured in liver-specific 11β-HSD1 knockout (LKO), *Hsd11b1* floxed (Control), complete global 11β-HSD1 deficient (Del), and 'old' hypomorphic 11β-HSD1 knockout (HSD1 KO) mice that underwent GTT. Data were analysed using one-way ANOVA with Dunnett's multiple comparisons test; n=6-19/group.

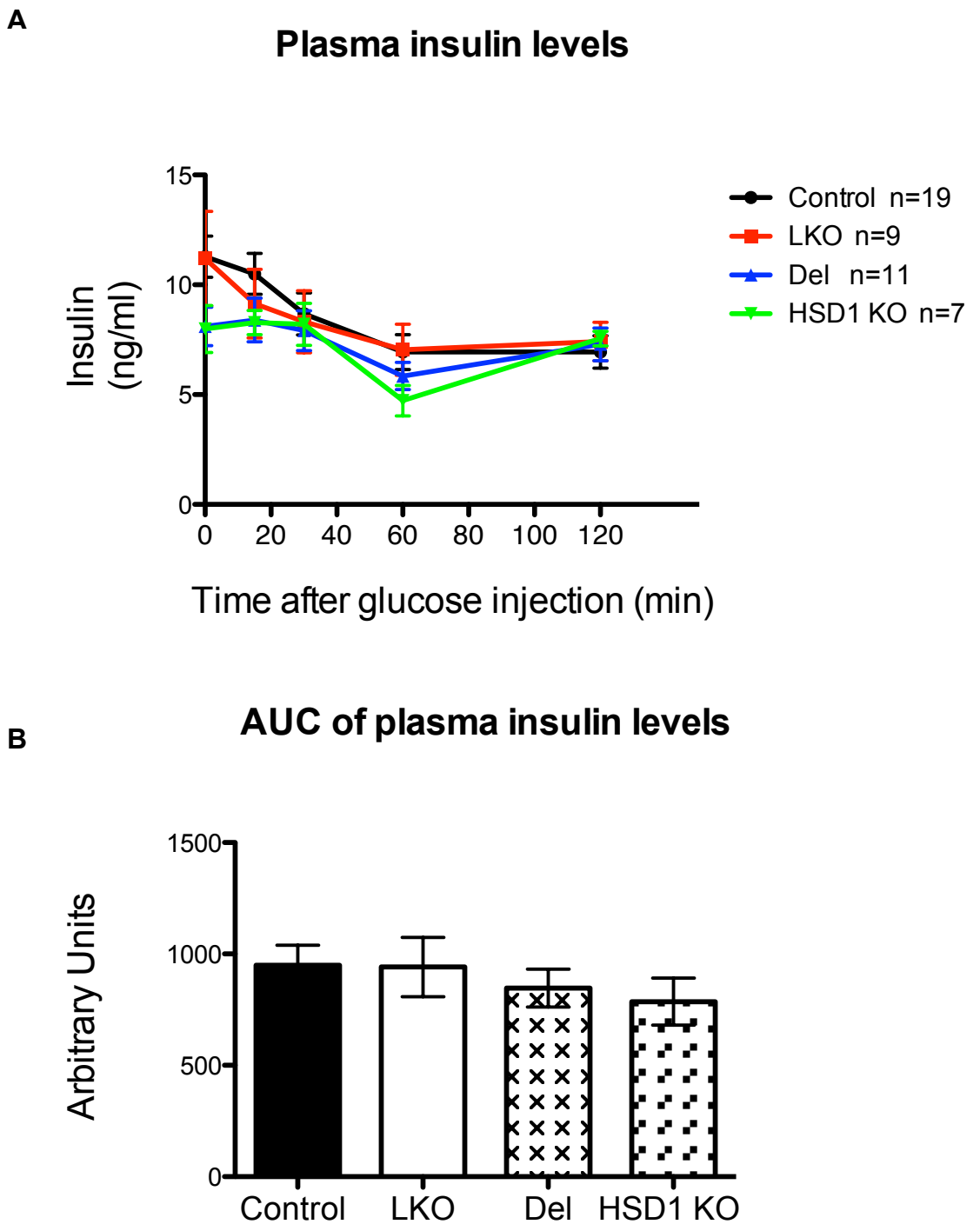


Figure 6.7: Similar plasma insulin levels during GTT in LKO, control, Del and HSD1 KO mice.

During the experiment described in Figure 6.5, **(A)** plasma insulin levels were measured in liver-specific 11β-HSD1 knockout (LKO), *Hsd11b1* floxed (Control), complete global 11β-HSD1 deficient (Del), and hypomorphic 11β-HSD1 knockout (HSD1 KO) mice that underwent GTT. Data were analysed using two-way repeated measures ANOVA with Bonferroni posttest; n=7-19/group. **(B)** Area under curve (AUC) of insulin levels during GTT between genotypes. Data were analysed using one-way ANOVA with Tukey's multiple comparisons test; n=7-19/group.

6.3.4. Similar hepatic *Srebp2*, *Hmgcr* and *Hmgcs* mRNA levels in HF-fed LKO and control mice

To investigate the effects of liver-specific 11β-HSD1 deficiency and HF feeding on cholesterol synthesis, hepatic *Srebp2*, *Hmgcr* and *Hmgcs* mRNA levels were measured.

Del mice were omitted from the gene expression analysis in this chapter as they showed extremely low levels of hepatic 18S rRNA as well as very low hepatic *Actb* and *Hprt* mRNA levels (data not shown). Although RNA extractions for this study were carried out in batches, at any particular time, there were liver samples from all genotypes, chosen at random. Therefore, it is likely that RNA was degraded in Del liver samples prior to extraction. Due to time restraint, RNA extractions were not repeated from Del liver samples.

HF-fed LKO mice exhibited no differences in hepatic *Srebp2* (Figure 6.8), *Hmgcr* (Figure 6.9A) or *Hmgcs* (Figure 6.9B) mRNA levels compared to HF-fed floxed *Hsd11b1* controls, suggesting no difference in cholesterol synthesis between genotypes. These findings are consistent with those from chow-fed LKO mice (Chapter 5). In this HF study, HSD1 KO mice also displayed no significant differences in hepatic *Srebp2* (Figure 6.8) and *Hmgcs* (Figure 6.9B) mRNA levels compared to control mice. However, significantly increased *Hmgcr* mRNA levels were observed in livers of HF-fed HSD1 KO mice compared to HF-fed control mice (Figure 6.9A).

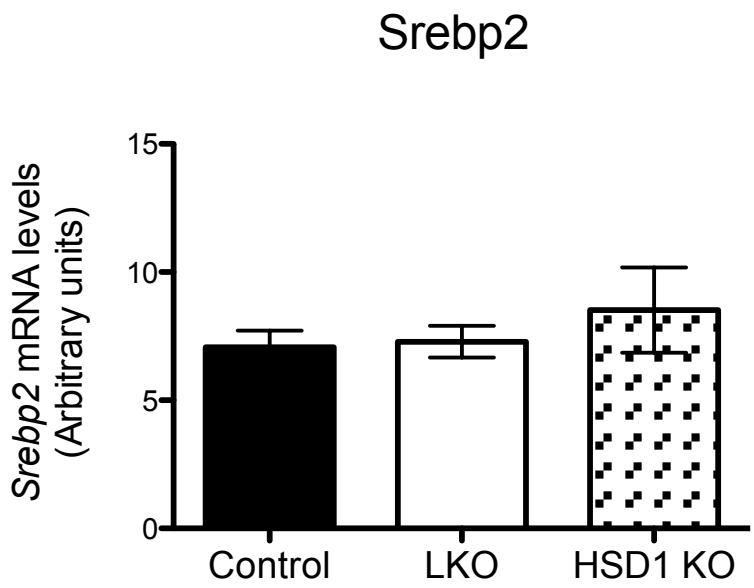


Figure 6.8: Similar hepatic *Srebp2* mRNA levels in LKO, control and HSD1 KO mice.

Total RNA was extracted from livers of male liver-specific 11β-HSD1 knockout (LKO), *Hsd11b1* floxed (Control) and hypomorphic 11β-HSD1 knockout (HSD1 KO) mice that were fed high fat diet for 14 weeks, from 8-10 weeks of age. Hepatic *Srebp2* mRNA levels were measured by qPCR. Levels of *Srebp2* are expressed relative to the average of *Hprt* and *Actb* mRNA levels, used as internal controls. Values are means ± SEM and were analysed by one-way ANOVA with Tukey's multiple comparisons test; n=7-9/group.

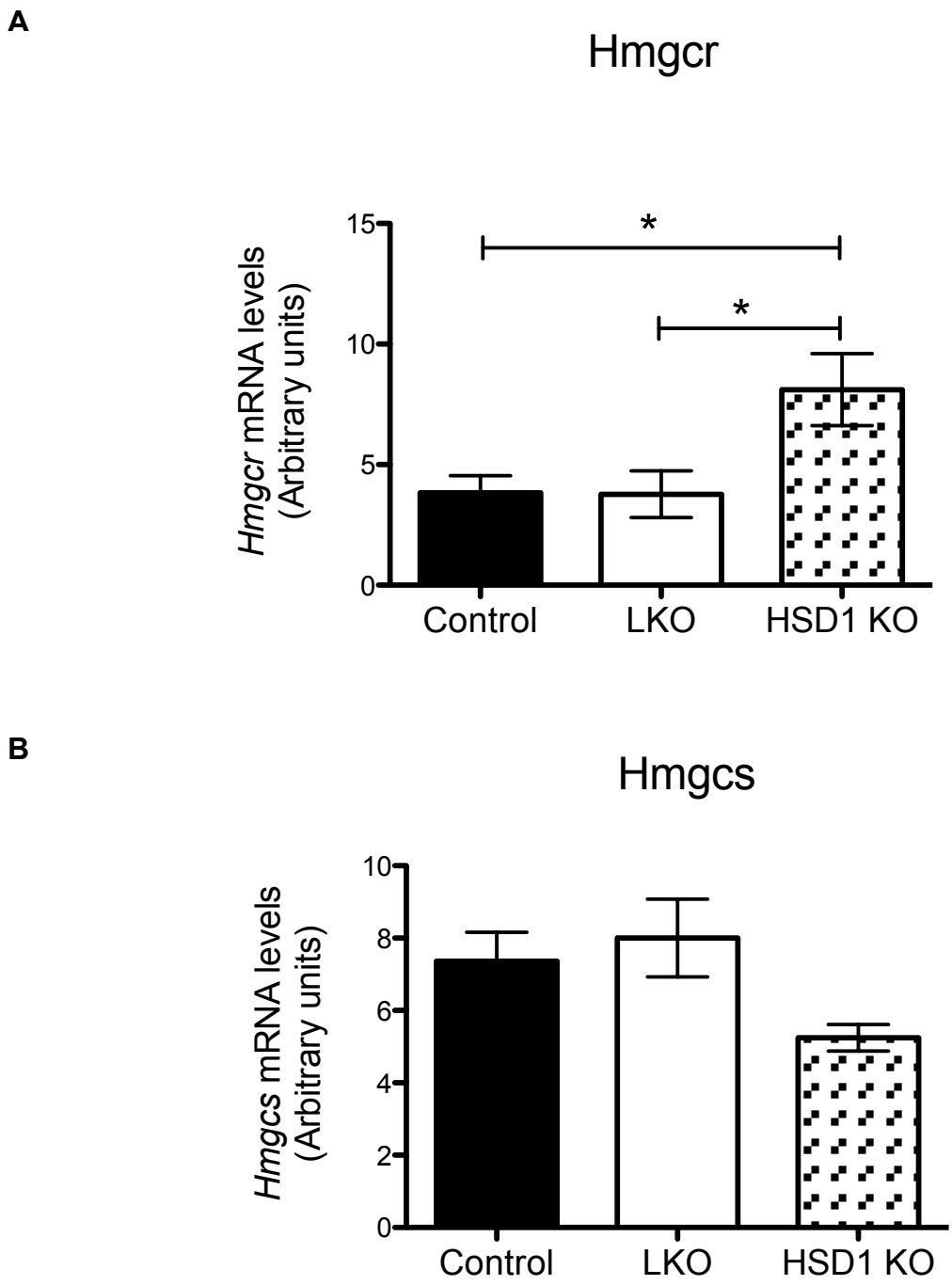


Figure 6.9: Similar levels of hepatic *Hmgcr* and *Hmgcs* mRNA in HF-fed LKO mice and control mice.

Total RNA was extracted from livers of male, 8-10 week old liver-specific 11β-HSD1 knockout (LKO), *Hsd11b1* floxed (Control) and original hypomorphic 11β-HSD1 knockout (HSD1 KO) mice that were fed high fat diet for 14 weeks. Messenger RNA levels were measured by qPCR. **(A)** *Hmgcr* and **(B)** *Hmgcs* mRNA levels are expressed relative to the average of *Hprt* and *Actb* mRNAs, used as internal controls. Data are means ± SEM and were analysed by one-way ANOVA with Tukey's multiple comparisons test; n=7-9/group; * $p<0.05$.

6.3.5. Similar hepatic *Lxrα*, *Cyp7a1*, *Abcg5* and *Abcg8* mRNA levels in HF-fed LKO and control mice, but reduced *Abcg5* mRNA levels in HSD1 KO mice compared to controls

Hepatic *Lxrα* mRNA expression is elevated in LOE mice (Chapters 3 and 4), with a similar pattern in HF-fed LOE mice (when compared to wild-type mice on the same diet) though this observation did not reach significance (see Figure 4.8, Chapter 4). To examine whether liver-specific 11β-HSD1 deficiency combined with HF feeding reduced hepatic LXRα expression, *Lxrα* mRNA levels were measured in livers of the HF-fed LKO, floxed *Hsd11b1* and HSD1 KO mice. Unlike LOE mice, there were no significant differences in hepatic *Lxrα* mRNA expression between LKO, control and HSD1 KO mice (Figure 6.10). Interestingly, there was a trend for reduced hepatic *Lxrα* mRNA levels in HSD1 KO mice compared to controls (Figure 6.10).

To investigate the effects of liver-specific 11β-HSD1 deficiency combined with HF feeding on cholesterol efflux and excretion, LXRα targets in these processes were examined; hepatic *Cyp7a1*, *Abcg5* and *Abcg8* mRNA levels were measured.

Consistent with the lack of effect of hepatic 11β-HSD1 on hepatic *Lxrα* expression, target genes involved in cholesterol degradation (*Cyp7a1*) and hepatobiliary cholesterol excretion (*Abcg5* and *Abcg8*) were unaltered in livers of HF-fed LKO mice compared to controls on the same diet (Figures 6.11 and 6.12). Surprisingly, *Abcg5* mRNA expression was significantly reduced in livers of HF-fed HSD1 KO mice compared to HF-fed controls (Figure 6.12A), with a similar trend in *Abcg8* mRNA (Figure 6.12B).

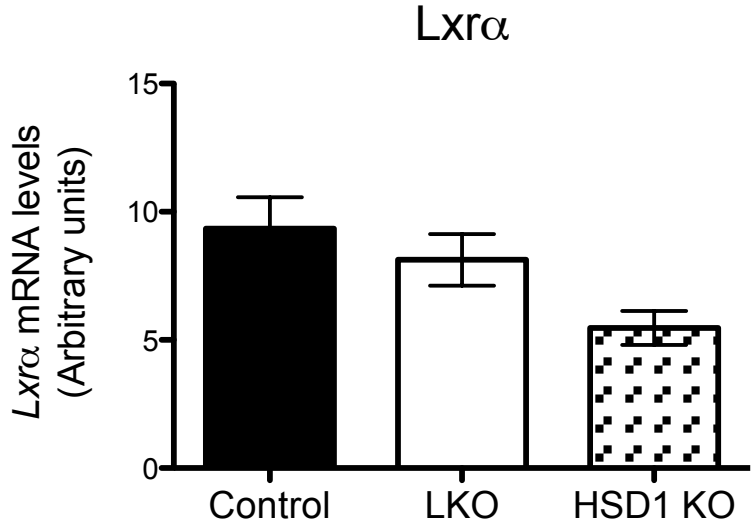


Figure 6.10: Similar hepatic *Lxrα* mRNA levels in LKO and control mice.

Total RNA was extracted from livers of male liver-specific 11β-HSD1 knockout (LKO), *Hsd11b1* floxed (Control) and original hypomorphic 11β-HSD1 knockout (HSD1 KO) mice, which were challenged with a high fat diet for 14 weeks from 8-10 weeks of age. Hepatic *Lxrα* mRNA levels were measured by qPCR. Levels of *Lxrα* are expressed relative to the average of *Hprt* and *Actb* mRNA levels, used as internal controls. Values are means ± SEM and were analysed by one-way ANOVA with Tukey's multiple comparisons test; n=7-9/group.

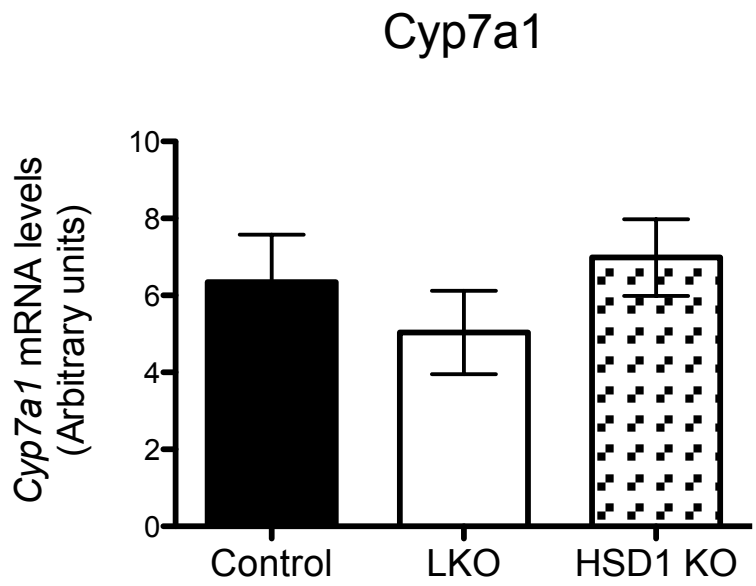


Figure 6.11: Similar levels of hepatic *Cyp7a1* mRNA in LKO, control and HSD1 KO mice.

Total RNA was extracted from livers of male, 8-10 week old liver-specific 11β-HSD1 knockout (LKO), *Hsd11b1* floxed (Control) and hypomorphic 11β-HSD1 knockout (HSD1 KO) mice that were fed high fat diet for 14 weeks. Levels of hepatic *Cyp7a1* mRNA were measured by qPCR and are expressed relative to the average of *Hprt* and *Actb* mRNAs, used as internal controls. Data are means ± SEM and were analysed by one-way ANOVA with Tukey's multiple comparisons test; n=7-9/group.

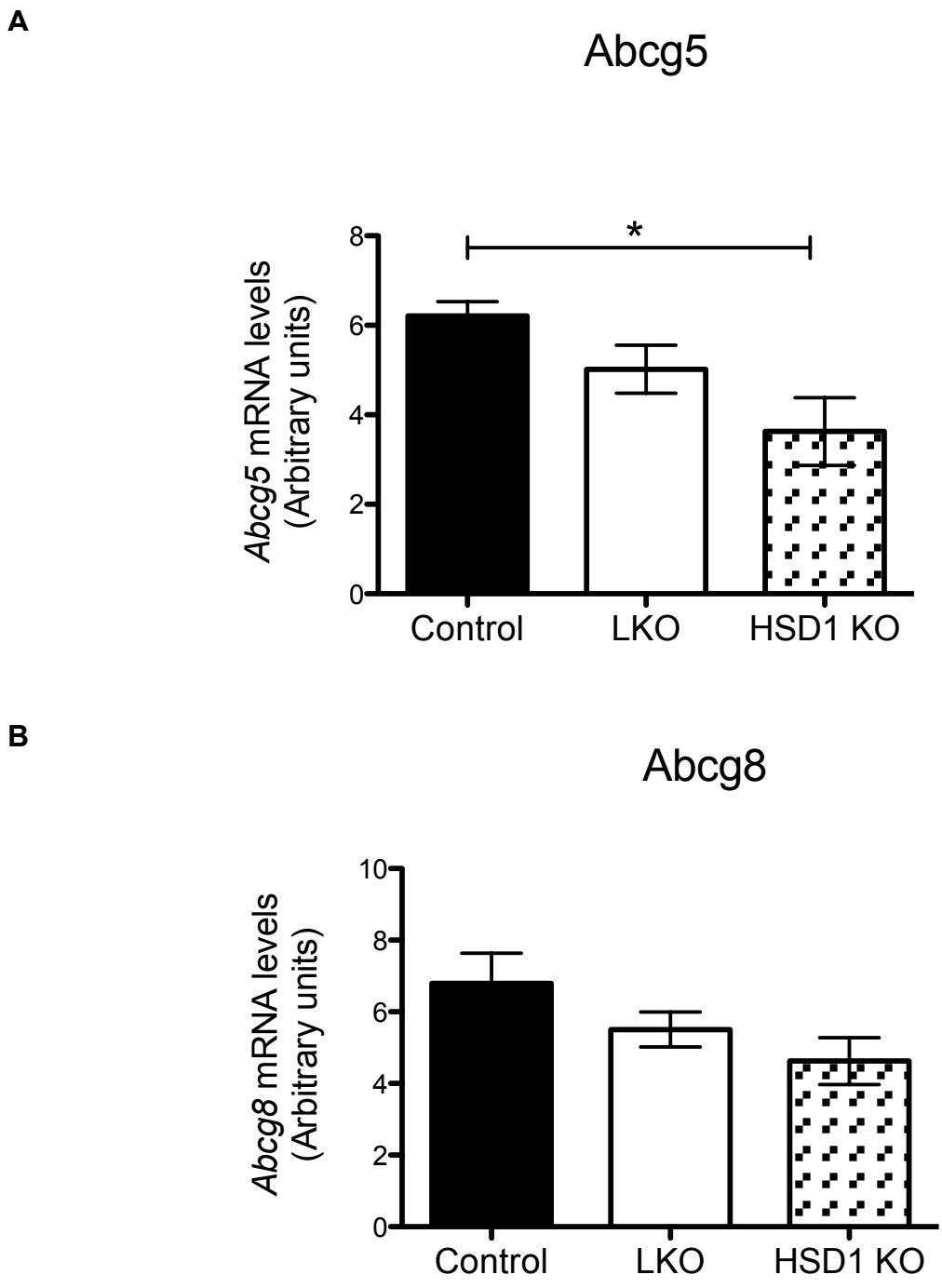


Figure 6.12: Similar hepatic *Abcg5* and *Abcg8* mRNA levels in LKO and control mice, but reduced *Abcg5* mRNA levels in HSD1 KO mice compared to controls.

Total RNA was extracted from livers of male liver-specific 11β-HSD1 knockout (LKO), *Hsd11b1* floxed (Control) and hypomorphic 11β-HSD1 knockout (HSD1 KO) mice, which were fed high fat diet for 14 weeks from 8-10 weeks of age. Messenger RNA levels were measured by qPCR. **(A)** *Abcg5* and **(B)** *Abcg8* mRNA levels are expressed relative to the average of *Hprt* and *Actb* mRNA levels, used as internal controls. Values are means ± SEM and were analysed by one-way ANOVA with Tukey's multiple comparisons test; n=7-9/group; * $p<0.05$.

6.4. Discussion

With 14 weeks of HF diet, liver-specific 11β-HSD1 deficiency did not alter body weight gain, liver weight, or adrenal gland weight. Unexpectedly, adipose tissue weight was reduced in HF-fed LKO mice, despite no effect on adipose tissue weight in HSD1 KO or Del mice. This effect of LKO on adipose tissue weight depends on HF diet as there were no differences in adiposity between chow-fed LKO and control mice (Chapter 5). These data differ from those of Lavery *et al*. (2012), who reported no difference in epididymal fat weights between their line of liver-specific 11β-HSD1 knockout mice and control mice after 18 weeks of HF diet (Lavery *et al*., 2012). Other fat depot weights were not reported. The discrepancy between the two studies may be a result of background strain differences: Lavery *et al.* (2012) generated their line on a mixed C57BL/6/129SvJ background (Lavery *et al*., 2012) whereas the LKO mice used in the current study are on a pure C57BL/6 strain background. Strain background can potently influence the response to HF diet-induced obesity (Black *et al*., 1998; Morton *et al*., 2004) as well as govern HPA responses to 11β-HSD1 deletion (Carter *et al*., 2009).

Reduced adiposity with liver-specific knockdown of 11β-HSD1 has clinical implications as inhibition of hepatic 11β-HSD1 may be beneficial in diet-induced adiposity. This finding suggests a crosstalk between liver and fat and makes it likely that hepatic 11β-HSD1 plays a role in adipose tissue lipogenesis/lipolysis or lipid storage/transport, at least in diet-induced obesity. Further work is needed to explore the mechanisms involved. In this study, there were no differences in subcutaneous adipocyte hypertrophy between LKO and control mice. However, a small number of samples were examined and it is worth repeating this investigation with a greater number of subcutaneous fat samples. Moreover, reduced adiposity could also be a result of decreased number of adipocytes and it would be interesting to investigate this in the subcutaneous and epididymal fat depots of HF-fed LKO. Additionally, gene expression in the adipose tissue of HF-fed LKO mice could be examined. A recent study by Beaven *et al*. (2013) showed that LXRs, at least in the setting of obesity, act as physiological suppressors of the SREBP-1c and ChREBP-β lipogenic

pathways in adipose tissue (Beaven *et al*., 2013). It would be interesting to investigate whether liver-specific 11β-HSD1 deficiency, combined with HF feeding, results in increased LXR expression in adipose tissue, thus resulting in a suppression of the above-mentioned lipogenic pathways and decreased adipose tissue lipogenesis. To study this further, expression of *Lxr* and its target genes involved in lipogenesis (e.g. *Srebp1c* and *Fas*) could be measured in adipose tissue (specifically subcutaneous and epididymal fat depots) from HF-fed LKO mice.

LKO mice did not show the decreased body weight gain that was observed in Del and HSD1 KO mice towards the end of the 14-week HF diet. This suggests that reduced body weight gain with 11β-HSD1 deficiency is not due to liver 11β-HSD1. Additionally, it is worth noting that despite decreased body weight gain, no differences were found in the final body weights (at the time of cull) between Del, or HSD1 KO, and control mice (data not shown). These results differ from previous data in globally 11β-HSD1 deficient mice, which revealed decreased body weight as well as reduced mesenteric fat with HF feeding, compared to control mice (Morton *et al*., 2004; Wamil *et al*., 2011). It is plausible that this discrepancy may reflect the use of different controls between the studies- floxed *Hsd11b1* mice (correct controls for LKO mice in the current study) versus externally-sourced C57BL/6 controls (in the published studies). Floxed *Hsd11b1* mice may exhibit some reduction in 11β-HSD1 activity, contributing to different findings between the studies (Zhang, 2010). Nevertheless, in another study using HSD1 KO mice, Michailidou *et al*. (2012) did not find any differences in weight gain, final terminal body weights or adiposity between HF-fed HSD1 KO mice and C57BL/6 controls (bred in-house) (Michailidou *et al*., 2012).

Fibrosis in subcutaneous fat was previously shown to be attenuated in HF-fed HSD1 KO mice compared to C57BL/6 controls (Michailidou *et al*., 2012). In this study, HSD1 KO mice showed reduced collagen deposition in subcutaneous fat compared to controls though this observation did not reach significance. LKO mice showed similar levels of collagen deposition in subcutaneous fat to control mice, suggesting that liver 11β-HSD1 deficiency (alone) does not contribute to the reduction in adipose tissue fibrosis in 11β-HSD1 deficient mice. Del mice exhibited increased collagen

deposition in their subcutaneous fat compared to HSD1 KO mice. Interestingly, evidence shows that HSD1 KO mice exhibit increased lung fibrosis following bleomycin injury (Yang, 2010) as well as elevated hepatic collagen deposition and increased liver fibrosis following carbon tetrachloride treatment (Zou 2013). It is worth noting that the principal difference between HSD1 KO and Del mice is that HSD1 KO mice have ~30% residual activity of 11β-HSD1 in tissues that use the P1 promoter of *Hsd11b1*. Importantly, this includes fibroblasts. In contrast, Del mice are a complete knockout of 11β-HSD1 (unpublished data, Professor Karen Chapman). It is possible that the residual 11β-HSD1 activity in fibroblasts (which use the P1 promoter) in HSD1 KO mice accounts for the differences in subcutaneous collagen content between HF-fed Del and HSD1 KO mice. Within the genotypes investigated in this study, Del mice are the only ones without fibroblast 11β-HSD1 expression, and it would be worth investigating whether Del mice on chow diet show increased adipose collagen deposition. Adipose tissue hypoxia gives rise to adipose fibrosis (Hosogai *et al*., 2007; Ye *et al*., 2007), and it has been shown that transgenic overexpression of the hypoxia-inducible factor 1α (HIF-1α) in adipose of *ob/ob* mice exacerbates collagen deposition and fibrosis compared to controls (Halberg *et al*., 2009). Therefore, it would also be interesting to study hypoxia in the adipose tissue of Del mice; for instance, is the expression of *Hif-1α* elevated in adipose tissue of Del mice?

Previous data revealed improved glucose tolerance and greater insulin sensitivity in HSD1 KO mice (Morton *et al*., 2004). In the current study, there were similar glucose levels during GTT in LKO and control mice. Furthermore, LKO mice showed an increased AUC for the GTT glucose levels compared to HSD1 KO mice. These data suggest that the improved glucose tolerance previously observed in HSD1 KO mice is not due to liver 11β-HSD1. Although Lavery *et al*. (2012) observed a mild improvement in glucose tolerance, insulin sensitivity was unaltered in their line of liver-specific 11β-HSD1 deficient mice (Lavery *et al*., 2012). In the current study, there were no differences in plasma insulin levels during GTT between LKO and other genotypes, and fasting insulin levels appeared to be extremely high in LKO mice (as well as in the global knockouts). Nonetheless, for a true measure of insulin sensitivity, an insulin tolerance test (ITT) needs to be carried out. Recent work by

Harno *et al.* (2013) demonstrated that while administration of 11-dehydrocorticosterone (11-DHC; substrate for 11β-HSD1) caused marked insulin resistance in wild-type controls, liver-specific 11β-HSD1 knockout mice given 11-DHC were protected from glucose intolerance and hyperinsulinaemia (Harno *et al*., 2013). However, a very recent study by Morgan *et al*. (2014) failed to observe any protection from glucose intolerance and hyperinsulinaemia in GC-treated liver-specific (or even adipose-specific) 11β-HSD1 knockout mice (Morgan *et al*., 2014). The reasons for the discrepancies between these studies is not clear, though the latter study, along with the findings from Lavery *et al*. (2012) and this study, suggest that hepatic 11β-HSD1 plays a relatively minor role in glucose homeostasis. Our adipose- and macrophage-specific 11β-HSD1 deficient mice in this 14-week HF diet experiment did not appear to be protected from glucose intolerance (unpublished data, Professor Karen Chapman), suggesting that 11β-HSD1 deficiency in adipose tissue or macrophages (alone) do not contribute to the improved glucose tolerance phenotype in globally 11β-HSD1 deficient mice. Previous data reveal a role for muscle 11β-HSD1 in insulin signalling (Morgan *et al*., 2009) and it may be that 11β-HSD1 action in muscle is important in determining whole-body glucose homeostasis. Another possibility is that combined knockout of 11β-HSD1 in liver and adipose may be needed to recapitulate the glucose tolerance phenotype in HSD1 KO mice as these tissues are both highly enriched for 11β-HSD1 and there might be cross talk between liver and adipose 11β-HSD1 (see below).

Intriguingly, although both Del and HSD1 KO mice showed reduced fasting glucose levels, Del mice did not show improved glucose tolerance; the circulating glucose levels during GTT were not decreased in Del mice as they were in HSD1 KO mice. The reason for this difference in glucose tolerance between these knockout models is unclear. While HSD1 KO mice display 11β-HSD1 deficiency in liver, adipose, brain and macrophages, there might be *Hsd11b1* expression in lymphocytes (as the P1 promoter is used in lymphocytes) (unpublished data, Professor Karen Chapman). It is worth exploring whether the discrepancies between HSD1 KO and Del mice are due to 11β-HSD1 expression and activity in lymphocytes or another cell type that uses the P1 promoter, including fibroblasts. In addition, whereas Del mice have exon 3 of *Hsd11b1* removed and only loxP sites added, the old HSD1 KO mice contain all

genomic DNA of *Hsd11b1* locus (some of it even duplicated) and have a large cassette and powerful promoter transcribing in the opposite direction to *Hsd11b1*. It is plausible that the construct, as well as the strong promoter in the cassette, may affect neighbouring genes.

Lack of differences in hepatic *Srebp2*, *Hmgcr* and *Hmgcs* mRNA levels between HF-fed LKO and control mice suggests that hepatic 11β-HSD1 deficiency, combined with HF feeding, does not alter cholesterol synthesis. These results are consistent with a study that reported unchanged hepatic *Srebp2* and *Hmgcr* mRNA expression following antisense oligonucleotide knock down of 11β-HSD1 in livers of C57BL/6 mice fed a Western-type diet with increased fat and cholesterol (Li *et al*., 2011). Similarly, Lavery *et al*. (2012) failed to observe any differences in hepatic *Hmgcr* mRNA levels between their liver-specific 11β-HSD1 knockout mice and controls (Lavery *et al*., 2012). These results also corroborate the findings in chow-fed LKO mice (Chapter 5). Data from LKO as well as LOE mice (Chapters 3 and 4) in this study indicate that hepatic 11β-HSD1 does *not* play a role in hepatic cholesterol synthesis.

In contrast with LOE mice, which showed increased hepatic *Lxrα* mRNA expression on both chow and WD (Chapters 3 and 4), there were no differences in *Lxrα* mRNA levels between livers of LKO and control mice. Consistent with this, Li *et al*. (2011) did not report differences in hepatic *Lxrα* mRNA levels in C57BL/6 mice with antisense-mediated inhibition of 11β-HSD1 (Li *et al*., 2011). In the current study, consistent with the lack of effect of liver 11β-HSD1 on *Lxrα* mRNA expression, LXR target genes involved in biliary cholesterol secretion, *Abcg5* and *Abcg8*, were unaltered in livers of HF-fed LKO mice compared to controls. This differs from the study by Li *et al*. (2011) that reported reduced *Abcg5* and *Abcg8* mRNA expression in liver of their experimental mice (Li *et al*., 2011). However, it was noted that the antisense oligonucleotide used in their study knocked down 11β-HSD1 not only in liver, but also in adipose tissue and lungs, therefore making the experimental model a partial global knockout of 11β-HSD1. In support of this, our data showed significantly decreased hepatic *Abcg5* mRNA expression in HSD1 KO mice, with a similar trend (though not significant) in hepatic *Abcg8* mRNA levels. Differences

between HF-fed HSD1 KO and LKO mice in this study suggest that the cause of reduced hepatic *Abcg5* and *Abcg8* mRNA levels in HSD1 KO mice may lie elsewhere than hepatocytes, and this requires further examination.

It is worth noting that mRNA levels in this dietary experiment are different to those in a previous dietary experiment; for instance, *Srebp2* mRNA levels in HF-fed control mice (in this experiment) are not similar to *Srebp2* mRNA levels in HF-fed WT mice in a previous experiment (Chapter 4). This might be due to the use of different controls in the two experiments; floxed *Hsd11b1* mice in this study versus C57BL/6 WT controls in the previous study. Also, factors such as different animal facilities (Little France animal facility for this study versus Hugh Robson Building animal unit for the dietary experiment in Chapter 4) or stress might also account for the above-mentioned differences.

Evidence suggests that knockout and pharmacological inhibition of 11β-HSD1 can reduce hepatic steatosis (Berthiaume *et al*., 2007; Berthiaume *et al*., 2010; Li *et al*., 2011). However, it is worth noting that both selective 11β-HSD1 inhibitors and small interfering RNA technology are not entirely selective with respect to target tissue and decrease 11β-HSD1 activity in both liver and fat. Morgan *et al.* (2014) showed that GC-treated liver-specific 11β-HSD1 knockout mice were not protected against metabolic disease including hepatic steatosis but GC-treated adipose-specific 11β-HSD1 knockout mice were protected from hepatic TG accumulation, increased serum free fatty acids (FFA) as well as from increased expression of adipose lipolytic enzymes (Morgan *et al*., 2014). These findings suggest that increased GC availability in adipose tissue (not liver) play a key role in hepatic steatosis. Various processes contribute to lipid accumulation within the liver, including *de novo* lipogenesis as well as FFA delivery from adipose tissue with subsequent re-esterification into TG. GCs are known to directly stimulate adipose tissue lipolysis, resulting in increased serum FFA levels (Xu *et al*., 2009). Therefore, in this model, decreased 11β-HSD1 in adipose tissue may limit lipolysis in this tissue, decreasing FFA availability and delivery to the Iiver, thereby reducing hepatic TG accumulation (Morgan *et al*., 2014). In support, HF-fed adipose-specific 11β-HSD1 knockout mice generated in our lab showed decreased liver weights compared to HF-fed control mice despite no

difference in liver weight of HF-fed HSD1 KO or Del mice (data not shown). It would be interesting to measure TG accumulation in livers from adipose-specific 11β-HSD1 knockout mice on this dietary experiment to investigate whether these adipose-specific 11β-HSD1 deficient mice are protected from hepatic steatosis.

In summary, there is no evidence that hepatic 11β-HSD1 plays a key role in cholesterol synthesis. Therefore, the data refute the original hypothesis. There is also no evidence that hepatic 11β-HSD1 deficiency, combined with HF feeding, alters cholesterol catabolism/excretion. These findings corroborate results from chow-fed LKO mice. Although it appears that the suggested role for hepatic 11β-HSD1 in hepatobiliary cholesterol secretion (Chapter 4) may be specific to liver 11β-HSD1 overexpression, HSD1 KO mice must be further investigated as they exhibit decreased hepatic *Abcg5* mRNA levels, with a similar trend for *Abcg8* mRNA. Finally, liver-specific 11β-HSD1 deficiency reduces adiposity upon HF feeding, suggesting that inhibition/deficiency of hepatic 11β-HSD1 may be advantageous in diet-induced adiposity. Further studies are required to unravel the mechanisms involved.

Chapter 7. General Discussion and Future Work

This thesis explored the role of hepatic 11β-HSD1 in cholesterol homeostasis. Alteration of cholesterol homeostasis is a major risk factor for atherosclerotic cardiovascular disease (Stamler *et al*., 2000; Weingartner *et al*., 2009; Tyler *et al*., 2009) and this work was encouraged by the increasing interest in 11β-HSD1 as a therapeutic target for atherosclerosis (Hermanowski-Vosatka *et al*., 2005; Walker, 2007). Chronic GC excess is associated with metabolic and cardiovascular disease, and 11β-HSD1 amplifies intracellular GC levels (Walker, 2007; Chapman *et al*., 2013). In addition, 11β-HSD1 catalyses the reduction of 7-KC to 7-βHC (Schweizer *et al*., 2004; Hult *et al*., 2004), and 7-KC has the potential to regulate cholesterol biosynthesis because it is an inhibitor of SREBP-2 cleavage (Brown *et al*., 2002). Hence, 11β-HSD1 may play an important role in cholesterol homeostasis but this area has been largely unexplored. Since liver is the key organ involved in cholesterol homeostasis as well as the organ with the highest expression of 11β-HSD1, the liver enzyme was chosen for study.

It was hypothesised that hepatic 11β-HSD1 promotes cholesterol biosynthesis. However, using models of liver 11β-HSD1 overexpression (LOE mice) and liver-specific 11β-HSD1 deficiency (LKO mice), data revealed that liver 11β-HSD1 does *not* promote cholesterol biosynthesis, at least through SREBP-2 mediated expression of mRNAs encoding hepatic cholesterol biosynthetic enzymes. Nevertheless, this study demonstrated increased hepatic *Abcg5* and *Abcg8* mRNA levels in LOE mice, suggesting a role for increased liver 11β-HSD1 in promoting hepatobiliary cholesterol secretion, thereby supporting a role for hepatic 11β-HSD1 in cholesterol homeostasis.

There is some evidence that LOE mice empty their gall bladders extremely efficiently whereas global deficiency of 11β-HSD1 impairs gall bladder emptying (unpublished data, Professor Karen Chapman). These findings might have implications for dietary lipid absorption; for instance, 11β-HSD1 deficiency (or inhibition) may reduce lipid absorption from the diet as bile release from gall bladder appears to be dependent on 11β-HSD1. It would be interesting to measure bile fluid and investigate this further in global 11β-HSD1 knockout as well as LKO and LOE mice. An interesting question that can be raised here is whether the role of 11β-HSD1 in cholesterol metabolism impacts on bile acid homeostasis or the other way round. A functional FXR binding

site has been identified in the *Abcg5* gene promoter and hepatic *Abcg5/8* expression was induced in mouse or human hepatocytes treated with FXR agonist or bile acids (Li *et al*., 2011b). The current study showed no evidence of altered bile acid synthesis in the mouse models investigated, and though not measured in this study, previously Paterson *et al*. (2004) reported no difference in hepatic *Fxr* mRNA levels between LOE and wild-type controls (Paterson *et al*., 2004). Nonetheless, GCs regulate bile acid homeostasis (Rose *et al*., 2011), and bile acids are not just competitive inhibitors of 11β-HSD1 (Escher *et al*., 1998; Ackermann *et al*., 1999) but also substrates (Odermatt *et al*., 2011), therefore, it is worth studying whether any of the metabolic effects of 11β-HSD1 deficiency are meditated through the potential role of 11β-HSD1 in bile acid metabolism. In this respect, another PhD study in the lab has shown that HSD1 KO mice exhibit a 100x decrease in the ratio of 7β:7α hydroxylated bile acids. Relevance of this remains unexplored and future studies are required.

It is important to dissect the impact of 11β-HSD1 inhibition/deficiency on cardiovascular events, the main cause of mortality in the phenotype of metabolic syndrome (Lloyd-Jones *et al*., 2009; Brown *et al*., 2010). As the final step in the reverse cholesterol transport pathway, hepatobiliary cholesterol secretion aids in the reduction of cholesterol accumulation within peripheral cells, including macrophage foam cells in atherosclerotic lesions (Ohashi *et al*., 2005; Wang and Rader, 2007; Dikkers and Tietge, 2010). Because hepatic ABCG5 and ABCG8 transporters are vital for cholesterol secretion into bile (Yu *et al*., 2002a; Yu *et al*., 2002b; Klett *et al*., 2004a; Plösch *et al*., 2004), it can be speculated that increased expression of these transporters in LOE mice might reduce atherosclerosis. When mice with overexpression of human ABCG5 and ABCG8 were crossed with atherosclerotic *Ldlr*$^{-/-}$ mice, the offspring developed significantly less atherosclerosis compared with wild-type control mice (Wilund *et al*., 2004). LOE mice have elevated levels of hepatic *Abcg5* and *Abcg8* mRNA levels and it is important to measure hepatobiliary cholesterol secretion in these mice, compared to wild-type controls. It would also be interesting to examine whether LOE mice crossed into the pro-atherogenic *Apoe*$^{-/-}$ background result in decreased atherosclerosis compared with *Apoe*$^{-/-}$ controls. However, preliminary data from breeding LOE mice with *Apoe*$^{-/-}$ mice showed no

effect on lesion size (unpublished data, Professor Karen Chapman), though hepatic gene expression has not been examined.

In addition to improving metabolic risk factors, recent findings suggest direct atheroprotective effects of 11β-HSD1 inhibition/deficiency. A study by Merck on 11β-HSD1 inhibition in *Apoe*$^{-/-}$ mice suggested reduced inflammatory gene expression in the vasculature as a protective mechanism against atherosclerosis (Luo *et al*., 2013). To directly examine the role of 11βHSD1 in atherosclerosis, recent studies used 11β-HSD1 knockout mice that were created on the *Apoe*$^{-/-}$ background (*Hsd11b1*$^{-/-}$*Apoe*$^{-/-}$ mice) (Garcia *et al*., 2013; Kipari *et al*., 2013). These studies showed that transplantation of bone marrow cells from *Hsd11b1*$^{-/-}$*Apoe*$^{-/-}$ mice confers atheroprotection to irradiated *Apoe*$^{-/-}$ mice, suggesting a key role for macrophage and/or other leukocyte 11β-HSD1 deficiency in the atheroprotective phenotype (Garcia *et al*., 2013; Kipari *et al*., 2013). Other important candidates include liver and adipose tissue, which are important tissues for cholesterol and lipid metabolism as well as abundant in 11β-HSD1. However, mice with liver- or adipose-specific 11β-HSD1 knockout on an *Apoe*$^{-/-}$ genetic background are not protected from atherosclerosis, whereas myeloid cell 11β-HSD1 deficiency on *Apoe*$^{-/-}$ background is atheroprotective (unpublished data, Professor Karen Chapman). Lipomics analysis of atherosclerotic plaques or foam macrophages from *Hsd11b1*$^{-/-}$/*Apoe*$^{-/-}$ versus *Apoe*$^{-/-}$ mice may help establish the involvement of macrophage 11β-HSD1 in atherosclerosis. To this end, data from Kipari *et al.* (2013) revealed increased ABCA1-dependent cholesterol efflux in HSD1 KO macrophages compared to C57BL/6 control mice (Kipari *et al*., 2013).

It is intriguing that data from LOE mice suggest a role for liver 11β-HSD1 in promoting hepatobiliary cholesterol secretion but data from Kipari *et al.* (2013) suggest that deficiency of 11β-HSD1 promotes cholesterol efflux from macrophages (Kipari *et al*., 2013). Thus, both liver overexpression of 11β-HSD1 and global 11β-HSD1 deficiency predict increased cholesterol removal, from liver to bile and from macrophages back to liver, respectively. It is interesting to speculate that 11β-HSD1 may have distinct functions in different tissues. LXR activation upregulates expression of cholesterol efflux transporters; ABCG5, ABCG8 in hepatocytes, and

ABCA1 in macrophages (Patel *et al*., 2008; Zhao and Wright, 2010; Hong *et al*., 2014). It has been shown that LXRα is required for reverse cholesterol transport and atheroprotection in *Apoe*$^{-/-}$ mice (Hong *et al*., 2012), and a very recent study showed that cholesterol efflux is dependent on LXRα, rather than LXRβ, in human macrophages (Ma *et al*., 2014). The current study suggests that, in liver, 11β-HSD1 promotes cholesterol efflux into the biliary lumen, possibly mediated through LXRα activation. GCs (down)regulate ABCA1 mRNA and protein levels in macrophages, thereby resulting in reduced cholesterol efflux from macrophages (Ayaori *et al*., 2006). I speculate that 11β-HSD1 in macrophages may suppress activation of LXRα, thereby preventing ABCA1-mediated cholesterol efflux from macrophages (Figure 7.1). Furthermore, LXR activation in macrophages attenuates inflammation (Joseph *et al*., 2003). Because inflammation and inflammatory signalling play a substantial role in atherosclerosis pathology (Tabas, 2010; Im and Osborne, 2011), suppression of LXR activation in macrophages would worsen atherosclerosis.

Another disease that is directly linked with biliary cholesterol secretion is gallstone disease. Unlike atherosclerotic cardiovascular disease where increased biliary cholesterol secretion is desirable, enhanced biliary cholesterol secretion is associated with biliary cholesterol super saturation, which increases risk of cholesterol gallstones (>90% of all gallstones) (Portincasa *et al*., 2006; Wang *et al*., 2009). In humans, specific mutations in ABCG5 and ABCG8 increase risk of cholesterol gallstone disease (Buch *et al*., 2007; Grunhage *et al*., 2007; Katsika *et al*., 2010). However, this has not been investigated in an experimental setting, which allows determination of cause-effect relationships. Measurement of cholesterol in the bile of wild-type, globally 11β-HSD1 deficient, liver-specific 11β-HSD1 deficient and liver 11β-HSD1 overexpressing mice would help to explore the involvement of 11β-HSD1 and liver 11β-HSD1 in biliary cholesterol secretion and cholesterol gallstone pathogenesis.

Although expected to decrease, hepatic *Lxrα*, *Abcg5* and *Abcg8* mRNA levels were unaltered with liver-specific 11β-HSD1 deficiency. Interestingly, global 11β-HSD1 deficiency, combined with HF diet, significantly decreased hepatic *Abcg5* mRNA levels, with a similar trend in *Abcg8* mRNA levels (as well as in hepatic *Lxrα* mRNA levels) compared to Cre^{-} *Hsd11b1* floxed controls. No side-by-side chow diet study

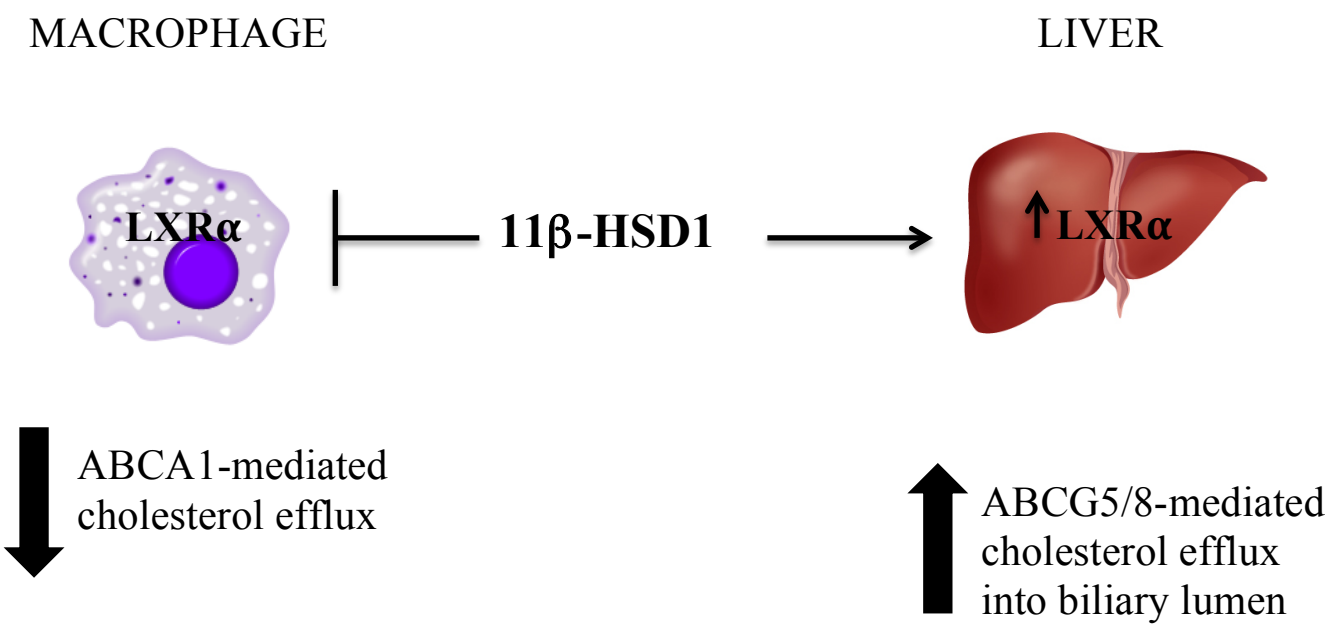


Figure 7.1: A hypothetical model of the distinct effects of 11β-HSD1 in liver and macrophages with respect to LXRα expression.

With respect to cholesterol metabolism, in liver, 11β-HSD1 results in LXRα activation, consequently leading to increased ABCG5/8-mediated hepatobiliary cholesterol secretion. However, in macrophages, 11β-HSD1 acts as a suppressor of LXRα activation, therefore decreasing ABCA1-mediated cholesterol efflux from macrophages.

was carried out as part of this experiment (Chapter 6), and therefore it is not entirely clear whether this is an effect of global 11β-HSD1 deficiency alone or is only manifest with 11β-HSD1 deficiency upon HF feeding. In a separate dietary experiment (Chapter 4), chow-fed HSD1 KO mice showed no differences in hepatic *Abcg5* and *Abcg8* mRNA levels compared to wild-type controls, therefore, it may be likely that effect of global 11β-HSD1 deficiency on *Abcg5* and *Abcg8* expression depends on HF feeding. It is important to note the use of different controls here. Cre^- floxed *Hsd11b1* mice were used as controls for studies with LKO mice (Chapters 5 and 6) and non-transgenic wild-type controls were compared to LOE transgenic mice (Chapters 3 and 4). These controls may not necessarily behave in the same way and there is now preliminary data showing that *Hsd11b1* floxed mice exhibit some reduction in 11β-HSD1 activity (unpublished data, Professor Karen Chapman). Therefore, it may be worthwhile confirming the above findings in LKO and HSD1 KO mice with wild-type controls as well as further investigating the consequence of the introduced LoxP sites upon expression of the 'floxed' *Hsd11b1* gene. More importantly, differences between HF-fed HSD1 KO and LKO mice in this study indicate that the cause of reduced *Lxrα*, *Abcg5* and *Abcg8* mRNA expression in HSD1 KO mice may lie elsewhere than hepatocytes, and this requires further examination.

Another significant (and unexpected) finding from this study is that liver-specific deficiency of 11β-HSD1 decreased subcutaneous and epididymal fat weight gain upon HF feeding compared to controls. This has implications for diet-induced adiposity and liver-specific 11β-HSD1 inhibition/deficiency may be advantageous in this respect. Further research is needed to unravel the mechanisms involved. Although no differences were observed in subcutaneous adipocyte hypertrophy, only a small number of samples (n=4-5/group) were examined and it is worth repeating this investigation with a greater number of subcutaneous fat samples as well as extending the study to epididymal fat. Additionally, LXRα expression in the adipose tissue of HF-fed LKO mice should be measured. Although well known as positive regulators of lipogenesis in liver (Repa *et al*., 2000; Schultz *et al*., 2000), a recent study by Beaven *et al*. (2013) established that LXRs act as physiological suppressors of the SREBP-1c and ChREBP-β lipogenic pathways in adipose tissue, specifically in the obese state (Beaven *et al*., 2013). It would be interesting to investigate whether

liver-specific 11β-HSD1 deficiency, combined with HF feeding, results in increased LXR expression in adipose tissue, thereby resulting in a suppression of the aforementioned lipogenic pathways and reduced adipose tissue lipogenesis. To investigate this further, adipose tissue from HF-fed LKO mice could be examined with respect to expression of *Lxr* and its target genes involved in lipogenesis (*Srebp1c*, *Fas* and *Scd1*). In addition, adipose tissue lipolysis should also be measured as LXRs have been implicated in lipolytic regulation as well as decreased fat cell size (Ross *et al*., 2002; Commerford *et al*., 2007).

This study does not distinguish whether the effects observed in the mouse models examined are dependent on metabolism of GCs or of 7-oxysterols by 11β-HSD1. It is known that 7-oxysterol substrates competitively inhibit GC metabolism and vice versa (Wamil *et al*., 2008; Balazs *et al*., 2009). In Chapter 4 of this thesis, liver and plasma samples from wild-type, LOE and HSD1 KO mice, which were fed chow, HF or WD, were sent to Professor William J. Griffith's laboratory (Swansea University, Wales, UK) for determination of oxysterol levels. However, the data were not returned in time for the submission of this thesis. Development of atherosclerotic plaques has been studied in adrenalectomised *Hsd11b1*$^{-/-}$*Apoe*$^{-/-}$ mice (with endogenous GCs removed) and it was suggested that both reactions of 11β-HSD1 might be involved in the atherosclerosis phenotype (Mitic *et al*., 2010).

While studies have highlighted the benefits of 11β-HSD1 inhibition, from a translation point of view, one of the important implications from this study is that in some cases (e.g. hypercholesterolaemia, atherosclerosis) increased hepatic 11β-HSD1 might be advantageous as it may promote secretion of cholesterol into bile. However, liver-specific 11β-HSD1 inhibition may be beneficial in diet-induced adiposity. Therefore, apart from its importance in pathogenesis, it is also important to fully resolve and appreciate the physiological functions of liver 11β-HSD1.

References

ABRASS C. K. (2004) Cellular lipid metabolism and the role of lipids in progressive renal disease. *Am J Nephrol.,* 24**,** 46-53.

ACKERMANN, D., VOGT, B., ESCHER, G., DICK, B., REICHEN, J., FREY, B. M. & FREY, F. J. (1999) Inhibition of 11beta-hydroxysteroid dehydrogenase by bile acids in rats with cirrhosis. *Hepatology*, 30, 623–629.

ACTON, S., RIGOTTI, A., LANDSCHULZ, K.T., XU, S., HOBBS, H.H. & KRIEGER, M. (1996) Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science*, 271, 518–520.

ADORNI, M.P., ZIMETTI, F., BILLHEIMER, J.T., WANG, N., RADER, D.J., PHILLIPS, M.C. & ROTHBLAT, G.H. (2007) The roles of different pathways in the release of cholesterol from macrophages. *J Lipid Res.*, 48, 2453–2462.

AGARWAL, A. K., MONDER, C., ECKSTEIN, B. & WHITE, P. C. (1989) Cloning and expression of rat cDNA encoding corticosteroid 11 beta-dehydrogenase. *J Biol Chem.* 264, 18939–18943.

AGARWAL, A.K., TUSIE-LUNA, M.T., MONDER, C., WHITE, P.C. (1990) Expression of 11 beta-hydroxysteroid dehydrogenase using recombinant vaccinia virus. *Mol Endocrinol.*, 4, 1827-1832

ALBERTI S., SCHUSTER G., PARINI P., FELTKAMP D., DICZFALUSY U., RUDLING M., ANGELIN B., BJORKHEM I., PETTERSSON S. & GUSTAFSSON J. A. (2001) Hepatic cholesterol metabolism and resistance to dietary cholesterol in LXRbeta-deficient mice. *J Clin Invest.,* 107, 565-573.

ALBERTS, P., ENGBLOM, L., EDLING, N., FORSGREN, M., KLINGSTROM, G., LARSSON, C., RONQUIST-NII, Y., OHMAN, B. & ABRAHMSEN L. (2002) Selective inhibition of 11beta- hydroxysteroid dehydrogenase type 1 decreases blood glucose concentrations in hyperglycaemic mice. *Diabetologia,* 45, 1528–1532.

ALTMANN, S.W., DAVIS, H.R. JR, YAO, X., LAVERTY, M., COMPTON, D.S., ZHU, L.J., CRONA, J.H., CAPLEN, M.A., HOOS, L.M., TETZLOFF, G., PRIESTLEY, T., BURNETT, D.A., STRADER, C.D. & GRAZIANO, M.P. (2002) The identification of intestinal scavenger receptor class B, type I (SR-BI) by expression cloning and its role in cholesterol absorption. *Biochim Biophys Acta.*, 1580, 77–93.

ALTMANN, S.W., DAVIS, H.R. JR, ZHU, L.J., YAO, X., HOOS, L.M., TETZLOFF, G., IYER, S.P., MAGUIRE, M., GOLOVKO, A., ZENG, M., WANG, L., MURGOLO, N. & GRAZIANO, M.P. (2004) Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. *Science*, 303, 1201–1204.

ANDREWS, R. C., ROOYACKERS, O. & WALKER B. R. (2003) Effects of the 11 beta-hydroxysteroid dehydrogenase inhibitor carbenoxolone on insulin sensitivity in men with type 2 diabetes. *J Clin Endocrinol Metab.,* 88, 285–291.

ANGELIN, B., PARINI, P. & ERIKSSON, M. (2002) Reverse cholesterol transport in man: promotion of fecal steroid excretion by infusion of reconstituted HDL. *Atheroscler Suppl.*, 3, 23–30.

ARAI, N., MASUZAKI, H., TANAKA, T., ISHII, T., YASUE, S., KOBAYASHI, N., TOMITA, T., NOGUCHI, M., KUSAKABE, T., FUJIKURA, J., EBIHARA, K., HIRATA, M., HOSODA, K., HAYASHI T., SAWAI, H., MINOKOSHI, Y., & NAKAO, K. (2007) Ceramide and adenosine 5′-monophosphate-activated protein kinase are two novel regulators of 11β-hydroxysteroid dehydrogenase type 1 expression and activity in cultured preadipocytes. *Endocrinology,* 148, 5268–5277.

ARIAS I.M., JAKOBY, W.B., POPPER, H., SCHACHTER, D. & SHAFRITZ, D. A. (2009) *The Liver: Biology and Pathobiology*, New York: Raven Press, pp 617–641.

ASSMANN, G. & GOTTO, A.M. (2004) HDL cholesterol and protective factors in atherosclerosis. *Circulation*, 109, 8-14.

ASZTALOS, B.F. & SCHAEFER, E.J. (2003) HDL in atherosclerosis: actor or bystander? *Atherosclerosis Supplements,* 4, 21-29.

ATANASOV, A. G., NASHEV, L. G., GELMAN, L., LEGEZA, B., SACK, R., PORTMANN, R. & ODERMATT, A. (2008) Direct protein-protein interaction of 11beta-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase in the endoplasmic reticulum lumen. *Biochim Biophys Acta.,* 1783: 1536–1543.

ATANASOV, A. G., NASHEV, L. G., SCHWEIZER, R. A., FRICK, C. & ODERMATT, A. (2004) Hexose-6-phosphate dehydrogenase determines the reaction direction of 11β-hydroxysteroid dehydrogenase type 1 as an oxoreductase. *FEBS Lett*, 571, 129–133.

AYAORI, M., SAWADA, S., YONEMURA, A., IWAMOTO, N., OGURA, M., TANAKA, N., NAKAYA, K., KUSUHARA, M., NAKAMURA, H. & OHSUZU, F. (2006) Glucocorticoid receptor regulates ATP-binding cassette transporter-A1 expression and apolipoprotein-mediated cholesterol efflux from macrophages. *Arterioscler Thromb Vasc Biol*., 26 (1), 163-168.

BAIGENT, C., KEECH, A., KEARNEY, P. M., BLACKWELL, L., BUCK, G., POLLICINO, C., KIRBY, A., SOURJINA, T., PETO, R., COLLINS, R., SIMES, R; Cholesterol Treatment Trialists' (CTT) Collaborators. (2005) Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. *Lancet*, 366 (9493):1267-78.

BALAZS, Z., NASHEV, L. G., CHANDSAWANGBHUWANA, C., BAKER, M. E. & ODERMATT A. (2009) Hexose-6-phosphate dehydrogenase modulates the effect of inhibitors and alternative substrates of 11beta-hydroxysteroid dehydrogenase 1. *Mol Cell Endocrinol*., 301: 117–122.

BANDSMA R. H., KUIPERS F., VONK R. J., BOVERHOF R., SAUER P. J., NAGEL G. T., ELZINGA H., NEESE R. A., HELLERSTEIN M. K. & STELLAARD F. (2000) The contribution of newly synthesized cholesterol to bile salt synthesis in rats quantified by mass isotopomer distribution analysis. *Biochim Biophys Acta*., 1483, 343-351.

BANHEGYI, G., BENEDETTI, A., FULCERI, R. & SENESI, S. (2004) Cooperativity between 11β-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase in the lumen of the endoplasmic reticulum. *J Biol Chem*. 279, 27017–27021.

BARTER, P.J., & RYE, K. A. (1996) High density lipoproteins and coronary heart disease. *Atherosclerosis*, 121, 1–12.

BASSO, F., FREEMAN, L., KNAPPER, C.L., REMALEY, A., STONIK, J., NEUFELD, E.B., TANSEY, T., AMAR, M.J., FRUCHART-NAJIB, J., DUVERGER, N., SANTAMARINA-FOJO, S. & BREWER, H.B. JR. (2003) Role of the hepatic ABCA1 transporter in modulating intrahepatic cholesterol and plasma HDL cholesterol concentrations. *J. Lipid Res.*, 44, 296-302.

BEAVEN S.W. & TONTONOZ P. (2006) Nuclear receptors in lipid metabolism: targeting the heart of dyslipidemia. *Annu Rev Med*., 57, 313-29.

BEAVEN, S. W., MATVEYENKO, A., WROBLEWSKI, K., CHAO, L., WILPITZ, D., HSU, T. W., LENTZ, J., DREW, B., HEVENER, A. L. & TONTONOZ, P. (2013) Reciprocal regulation of hepatic and adipose lipogenesis by liver X receptors in obesity and insulin resistance. *Cell Metab*.,18 (1), 106-17.

BEŁTOWSKI, J. (2008) Liver X Receptors (LXR) as therapeutic targets in dyslipidemia. *CARDIOVASC THER*., 26, 297–316.

BERG J. M., TYMOCZKO J. L. & STRYER L. (2002) The complex regulation of cholesterol biosynthesis takes place at several levels. *Biochemistry, (Section 26.3) Ed*. 5th, New York: W H Freeman.

BERGE, K.E., TIAN, H., GRAF, G.A., YU, L., GRISHIN, N.V., SCHULTZ, J., KWITEROVICH, P., SHAN, B., BARNES, R. & HOBBS, H.H. (2000) Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. *Science*, 290, 1771–1775.

BERTHIAUME, M., LAPLANTE, M., FESTUCCIA, W. T., BERGER, J. P., THIERINGER, R., & DESHAIES, Y. (2010) Preliminary report: pharmacologic 11beta-hydroxysteroid dehydrogenase type 1 inhibition increases hepatic fat oxidation in vivo and expression of related genes in rats fed an obesogenic diet. Metabolism, 59 (1), 114-7.

BERTHIAUME, M., LAPLANTE, M., FESTUCCIA, W., GÉLINAS, Y., POULIN, S., LALONDE, J., JOANISSE, DR, THIERINGER, R. & DESHAIES, Y. (2007) Depot-specific modulation of rat intraabdominal adipose tissue lipid metabolism by pharmacological inhibition of 11beta-hydroxysteroid dehydrogenase type 1. *Endocrinology*, 148 (5), 2391-7.

BEYEA, M. M., HESLOP, C. L., SAWYEZ, C. G., EDWARDS, J. Y., MARKLE, J. G., HEGELE, R. A. & HUFF, M. W. (2006) Selective up-regulation of LXR-regulated genes ABCA1, ABCG1 and APOE in macrophages through increased endogenous synthesis of 24(S), 25-epoxycholesterol. *J Biol Chem*., 282 (8), 5207–5216.

BHATTACHARYYA, A.K., CONNOR, W.E. & LIN, D.S. (1983) The origin of plant sterols in the skin surface lipids in humans: from diet to plasma to skin. *J Invest Dermatol.*, 80, 294-296.

BHATTACHARYYA, A.K., CONNOR, W.E. & SPECTOR, A.A. (1972) Excretion of sterols from the skin of normal and hypercholesterolemic humans. Implications for sterol balance studies. *J Clin Invest.*, 51, 2060-2070.

BIETRIX, F., YAN, D., NAUZE, M., ROLLAND, C., BERTRAND-MICHEL, J., COMERA, C., SCHAAK, S., BARBARAS, R., GROEN, A.K., PERRET, B., TERCE, F. & COLLET, X. (2006) Accelerated lipid absorption in mice overexpressing intestinal SR-BI. *J Biol Chem.*, 281, 7214–7219.

BJORKHEM, I., ANDERSSON, O., DICZFALUSY, U., SEVASTIK, B., XIU, R., DUAN, C. & LUND, E. (1994) Atherosclerosis and Sterol 27-Hydroxylase: Evidence for a role of this enzyme in elimination of cholesterol from human macrophages. *Proc Natl Acad Sci. USA.*, 91, 8592-8596.

BJÖRNTORP, P. & ROSMOND, R. (1999) Visceral obesity and diabetes. *Drugs*, 58, Suppl 1:13-8; discussion 75-82.

BLACK, B. L., CROOM, J., EISEN, E. J., PETRO, A. E., EDWARDS, C. L. & SURWIT, R. S. (1998) Differential effects of fat and sucrose on body composition in A/J and C57BL/6J mice. *Metabolism*, 47, 1354 –1359.

BLOCH, K., BERG, N. & RITTENBERG, D. (1943) The biologic conversion of cholesterol to cholic acid. J *Blot. Chem.*, 149, 511-517.

BODZIOCH, M., ORSO, E., KLUCKEN, J., LANGMANN, T., BÖTTCHER, A., DIEDERICH, W., DROBNIK, W., BARLAGE, S., BÜCHLER, C., PORSCH-OZCÜRÜMEZ, M., KAMINSKI, W.E., HAHMANN, H.W., OETTE, K., ROTHE, G., ASLANIDIS, C., LACKNER, K.J. & SCHMITZ, G. (1999) The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat Genet.*, 22, 347–351.

BOONE, L.R., BROOKS, P.A., NIESEN, M.I. & NESS, G.C. (2011) Mechanism of resistance to dietary cholesterol. *Journal of Lipids.,* 2011, Article ID 101242, 9.

BREWER, H. B. Jr. & SANTAMARINA-FOJO, S. (2003) Clinical significance of high-density lipoproteins and the development of atherosclerosis: focus on the role of the adenosine triphosphate-binding cassette protein A1 transporter. *Am J Cardiol*., 92 (4B), 10K-16K.

BRIAND F., NAIK S. U., FUKI I., MILLAR J. S., MACPHEE C., WALKER M., BILLHEIMER J., ROTHBLAT G. & RADER D. J. (2009) Both the peroxisome proliferator-activated receptor delta agonist, GW0742, and ezetimibe promote reverse cholesterol transport in mice by reducing intestinal reabsorption of HDL-derived cholesterol. *Clin Trans Sci*., 2, 127-33.

BROOKS-WILSON, A., MARCIL, M., CLEE, S.M., ZHANG, L.H., ROOMP, K., van DAM, M., YU, L., BREWER, C., COLLINS, J.A., MOLHUIZEN, H.O., LOUBSER, O., OUELETTE, B.F., FICHTER, K., ASHBOURNE-EXCOFFON, K.J., SENSEN, C.W., SCHERER, S., MOTT, S., DENIS, M., MARTINDALE, D., FROHLICH, J., MORGAN, K., KOOP, B., PIMSTONE, S., KASTELEIN, J.J., GENEST, J. JR & HAYDEN, M.R. (1999) Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat Genet.*, 22, 336–345.

BROWN A. J., SUN L. P., FERAMISCO J. D., BROWN M. S. & GOLDSTEIN J. L. (2002) Cholesterol addition to ER membranes alters conformation of SCAP, the SREBP escort protein that regulates cholesterol metabolism. *Mol Cell*., 10, 237-245.

BROWN M. S. & GOLDSTEIN J. L. (1980) Multivalent feedback regulation of HMG CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. *J Lipid Res.*, 21 (5), 505–517.

BROWN M. S. & GOLDSTEIN J. L. (1999) A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. *Proc Natl Acad Sci USA.,* 96, 11041- 11048.

BROWN, A., REYNOLDS, L.R. & BRUEMMER, D. (2010) Intensive glycemic control and cardiovascular disease: an update. *Nat Rev Cardiol*, 7, 369–375.

BROWN, M.S. & GOLDSTEIN, J.L. (1997) The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell*, 89, 331-340.

BRUFAU G., GROEN A. K. & KUIPERS F. (2011) Reverse cholesterol transport revisited: contribution of biliary versus intestinal cholesterol excretion. *Arterioscler Thromb Vasc Biol.*, 31, 1726–1733.

BRULEY, C., LYONS, V., WORSLEY, A. G., WILDE, M. D., DARLINGTON, G. D., MORTON, N. M, SECKL, J. R. & CHAPMAN, K. E. (2006) A novel promoter for the 11beta-hydroxysteroid dehydrogenase type 1 gene is active in lung and is C/EBPalpha independent. *Endocrinology*, 147, 2879–2885.

BRUNHAM, L.R., KRUIT, J.K., IQBAL, J., FIEVET, C., TIMMINS, J.M., PAPE, T.D., COBURN, B.A., BISSADA, N., STAELS, B., GROEN, A.K., HUSSAIN, M.M., PARKS, J.S., KUIPERS, F. & HAYDEN, M.R. (2006) Intestinal ABCA1 directly contributes to HDL biogenesis in vivo. *J Clin Invest.*, 116, 1052–1062.

BUCH, S., SCHAFMAYER, C., VÖLZKE, H., BECKER, C., FRANKE, A., VON ELLER-EBERSTEIN, H., KLUCK, C., BÄSSMANN, I., BROSCH, M., LAMMERT, F., MIQUEL. J. F., NERVI, F., WITTIG, M., ROSSKOPF, D., TIMM, B., HÖLL, C., SEEGER, M., ELSHARAWY, A., LU, T., EGBERTS, J., FÄNDRICH, F., FÖLSCH U. R, KRAWCZAK, M., SCHREIBER, S., NÜRNBERG, P., TEPEL, J. & HAMPE, J. (2007) A genome-wide association scan identifies the hepatic cholesterol transporter ABCG8 as a susceptibility factor for human gallstone disease. *Nat Genet.*, 39, 995–999.

BUCKINGHAM, J. C. (2006) Glucocorticoids: exemplars of multi-tasking. *Br J Pharmacol.,* 147 Suppl 1**,** S258-68.

BUETTNER, R., PARHOFER, K.G., WOENCKHAUS, M., WREDE, C.E., KUNZ-SCHUGHART, L.A., SCHÖLMERICH, J. & BOLLHEIMER, L.C. (2006) Defining high-fat-diet rat models: metabolic and molecular effects of different fat types. *J Mol Endocrinol.*, 36, 485-501.

BUJALSKA, I. J., DRAPER, N., MICHAILIDOU, Z., TOMLINSON, J. W, WHITE, P. C, CHAPMAN, K. E., WALKER, E. A. & STEWART, P. M. (2005) Hexose-6-phosphate dehydrogenase confers oxo-reductase activity upon 11β-hydroxysteroid dehydrogenase type 1. *J Mol Endocrinol*., 34, 675–684.

CAI, L., ECKHARDT, E.R., SHI, W., ZHAO, Z., NASSER, M., de VILLIERS, W.J. & van der WESTHUYZEN, D.R. (2004) Scavenger receptor class B type I reduces cholesterol absorption in cultured enterocyte CaCo-2 cells. *J Lipid Res.*, 45, 253–262.

CALKIN A. C. & TONTONOZ P. (2010) Liver x receptor signaling pathways and atherosclerosis. *Arterioscler Thromb Vasc Biol.*, 30 (8), 1513–1518.

CAREY M. C., SMALL D. M. & BLISS C. M. (1983) Lipid digestion and absorption. *Annu Rev Physiol*., 45, 651-77.

CARSTEA, E.D., MORRIS, J.A., COLEMAN, K.G., LOFTUS, S.K., ZHANG, D., CUMMINGS, C., GU, J., ROSENFELD, M.A., PAVAN, W.J., KRIZMAN, D.B., NAGLE, J., POLYMEROPOULOS, M.H., STURLEY, S.L., IOANNOU, Y.A., HIGGINS, M.E., COMLY, M., COONEY, A., BROWN, A., KANESKI, C.R., BLANCHETTE-MACKIE, E.J., DWYER, N.K., NEUFELD, E.B., CHANG, T.Y., LISCUM, L., STRAUSS, J.F. 3RD, OHNO, K., ZEIGLER, M., CARMI, R., SOKOL, J., MARKIE, D., O'NEILL, R.R., van DIGGELEN, O.P., ELLEDER, M., PATTERSON, M.C., BRADY, R.O., VANIER, M.T., PENTCHEV, P.G. & TAGLE, D.A. (1997) Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis. *Science*, 277, 228 –231.

CARTER, R. N, PATERSON, J. M., TWOROWSKA, U., STENVERS, D. J., MULLINS, J. J, SECKL, J. R., & HOLMES, M. C. (2009) Hypothalamic-pituitary-

adrenal axis abnormalities in response to deletion of 11β-HSD1 is strain-dependent. *J Neuroendocrinol*., 21, 879–887.

CHAMBERS, C.M. & NESS, G.C. (1997) Translational regulation of hepatic HMG-CoA reductase by dietary cholesterol. *Biochem Biophys Res Commun.*, 232, 278–281.
CHANG, T.Y., HASAN, M.T., CHIN, J., CHANG, C.C., SPILLANE, D.M. & CHEN, J. (1997) Chinese hamster ovary cell mutants affecting cholesterol metabolism. *Curr Opin Lipidol.*, 8, 65-71.

CHAPMAN K.E. & SECKL J.R. (2008) 11beta-HSD1, inflammation, metabolic disease and age-related cognitive (dys)function. *Neurochem Res*., 33 (4), 624-36.
CHAPMAN, K. E., HOLMES M. & SECKL, J. R. (2013). 11β-Hydroxysteroid Dehydrogenases: Intracellular Gate-Keepers of Tissue Glucocorticoid Action. *Physiological Reviews*, 93 (3), 1139-1206
CHAPPELL, D. A. & MEDH, J. D. (1998) Receptor-mediated mechanisms of lipoprotein remnant catabolism. *Prog Lipid Res*., 37, 393-422.

CHAWLA, A., BOISVERT, W. A., LEE, C. H., LAFFITTE, B. A., BARAK, Y., JOSEPH, S. B., LIAO, D., NAGY, L., EDWARDS, P. A., CURTISS, L. K., EVANS, R. M. & TONTONOZ, P. (2001) A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. *Mol Cell*.,7 (1), 161-71.

CHENG S. H. & STANLEY M. M. (1959) Secretion of cholesterol by intestinal mucosa in patients with complete common bile duct obstruction. *Proc Soc Exp Biol Med*., 101, 223- 225.

CHIANG, J.Y. (2004) Regulation of bile acid synthesis: pathways, nuclear receptors, and mechanisms. *J Hepatology*, 40, 539–551.

CHIANG, J.Y.L. & VLAHCEVIC, Z.R. (1996) *The regulation of cholesterol conversion to bile acids,* Greenwich: CT:JAI Press, Inc., pp 269-316.

CHINETTI, G., LESTAVEL. S., BOCHER, V., REMALEY, A. T., NEVE, B., TORRA, I. P., TEISSIER, E., MINNICH, A., JAYE, M., DUVERGER, N., BREWER, H. B., FRUCHART, J. C., CLAVEY, V. & STAELS, B. (2001) PPAR-alpha and PPAR-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. *Nat Med*., 7 (1), 53-8.

CHOWDHURY, R., WARNAKULA, S., KUNUTSOR, S., CROWE, F., WARD, H. A., JOHNSON, L., FRANCO, O. H., BUTTERWORT, A. S., FOROUHI, N. G., THOMPSON, S. G., KHAW, K., MOZAFFARIAN, D., DANESH, J. & ANGELANOTONIO, E. D. (2014) Association of Dietary, Circulating, and Supplement Fatty Acids With Coronary Risk: A Systematic Review and Meta-analysis. *Ann Intern Med*., 160, 398-406.

CLINKENBEARD K. D., SUGIYAMA T., REED W. D. & LANE M. D. (1975) Cytoplasmic 3-hydroxy-3-methylglutaryl coenzyme A synthase from liver.

Purification, properties, and role in cholesterol synthesis. *J Biol Chem*., 250, 3124-3135.

COMMERFORD, S. R., VARGAS, L., DORFMAN, S. E., MITRO, N., ROCHEFORD, E. C., MAK, P. A., LI, X., KENNEDY, P., MULLARKEY, T. L. & SAEZ, E. (2007) Dissection of the insulin-sensitizing effect of liver X receptor ligands. *Mol Endocrinol*., 21, 3002–3012.

COSTET, P., LUO, Y., WANG, N. & TALL, A.R. (2000) Sterol-dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor. *J Biol Chem.*, 275, 28240-28245.

COUTINHO A. E., BROWN J. K., YANG F., BROWNSTEIN D. G., GRAY M., SECKL J. R., SAVILL J. S. & CHAPMAN K. E. (2013) Mast cells express 11β-hydroxysteroid dehydrogenase type 1: a role in restraining mast cell degranulation. *PLoS ONE*, 8 (1), e54640.

CUCHEL, M. & RADER, D.J. (2006) Macrophage reverse cholesterol transport: key to the regression of atherosclerosis? *Circulation*, 113, 2548–2555.

CULLEN P, RAUTERBERG J & LORKOWSKI S. (2005) The pathogenesis of atherosclerosis. *Handb Exp Pharmacol*., 170, 3–70.

CURTISS, L.K. & BOISVERT, W.A. (2000) Apolipoprotein E and atherosclerosis. *Curr. Opin. Lipidol*., 11, 243-251.

DALLMAN, M. F., LA FLEUR, S. E., PECORARO, N. C., GOMEZ, F., HOUSHYAR, H. & AKANA S. F. (2004) Minireview: glucocorticoids--food intake, abdominal obesity, and wealthy nations in 2004. *Endocrinology*, 145, 2633-8.

DAM, H. (1958) Historical introduction to cholesterol. IN: COOK, R.P. (Eds.) *Chemistry, Biochemistry and Pathology*, New York, NY: Academic Press, pp 1–14.
DANIELS, T. F., KILLINGER, K. M., MICHAL, J. J., WRIGHT, R. W. JR, & JIANG, Z. (2009) Lipoproteins, cholesterol homeostasis and cardiac health. *Int J Biol Sci*., 5, 474-88.

DAVIS H. R. JR., ZHU L. J., HOOS L. M., TETZLOFF G., MAGUIRE M., LIU J., YAO X., IYER S. P., LAM M. H., LUND E. G., DETMERS P. A., GRAZIANO M. P. & ALTMANN S. W. (2002) Niemann-Pick C1 Like 1 (NPC1L1) is the intestinal phytosterols and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. *J Biol Chem*., 279, 33586-92.

DAVIS H.R. & VELTRI E. P. (2007) Zetia: Inhibition of Niemann-Pick C1 Like 1 (NPC1L1) to reduce intestinal cholesterol absorption and treat hyperlipidaemia. *J Atheroscler Throm*., 14, 99-108.

DE BACQUER, D., DE BACKER, G., OSTÖR, E., SIMON, J., PYÖRÄLÄ, K; EUROASPIRE I Study Group. (2003) Predictive value of classical risk factors and their control in coronary patients: a follow-up of the EUROASPIRE I cohort. *Eur J Cardiovasc Prev Rehabil.,* 10, 289-95.

DEBOSE-BOYD R. A., OU J., GOLDSTEIN J. L. & BROWN M. S. (2001) Expression of sterol regulatory element-binding protein 1c (SREBP-1c) mRNA in rat hepatoma cells requires endogenous LXR ligands. *Proc. Natl. Acad. Sci. USA*., 98, 1477–1482.

DENG, Q.G., SHE, H., CHENG, J.H., FRENCH, S.W., KOOP, D., XIONG, S. & TSUKAMOTO, H. (2005) Steatohepatitis induced by intragastric overfeeding in mice. *Hepatology*, 42, 905–914.

DE SOUSA PEIXOTO R. A., TURBAN S., BATTLE J. H., CHAPMAN K. E., SECKL J. R. & MORTON N. M. (2008) Preadipocyte 11beta-hydroxysteroid dehydrogenase type 1 is a keto-reductase and contributes to diet-induced visceral obesity in vivo. *Endocrinology,* 149, 1861–1868.

DIKKERS, A. & TIETGE, U.J. (2010) Biliary cholesterol secretion: More than a simple ABC. World *J Gastroenterol.*, 16, 5936-5945.

DIXON J. B. (2010) Mechanisms of chylomicron uptake into lacteals. *Ann N Y Acad Sci*., 1207 (Suppl 1), E52–E57.

DORFMAN, E.S., LAURENT, D. & GOUNARIDES, S.J. (2009) Metabolic implications of dietary trans-fatty acids. *Obesity*, 17, 1200-1207.

DRAPER, N. & STEWART, P. M. (2005) 11β-hydroxysteroid dehydrogenase and the pre-receptor regulation of corticosteroid hormone action. *J Endocrinol,* 186, 251-271.

DUANE W. C. & JAVITT N. B. (1999) 27-Hydroxycholesterol: production rates in normal human subjects. *J Lipid Res.,* 40*,* 1194–1199.

DUANE, W.C. & JAVITT, N.B. (1999) 27-Hydroxycholesterol: production rates in normal human subjects. *J. Lipid Res.*, 40, 1194–1199.

DUJOVNE C. A., ETTINGER M. P., MCNEER J. F., LIPKA L. J., LEBEAUT A. P., SURESH R., YANG B., VELTRI E. P.; Ezetimibe Study Group. (2002) Efficacy and safety of a potent new selective cholesterol absorption inhibitor, ezetimibe, in patients with primary hypercholesterolaemia. *Am J Cardiol*., 90, 1092-7.

DUPLUS, E., GLORIAN, M. & FOREST, C. (2000). Fatty acid regulation of gene transcription. *J Biol Chem.*, 275, 30749-30752.

DUSSAULT, I. & FORMAN, B.M. (2000) Prostaglandins and fatty acids regulate transcriptional signalling via the peroxisome proliferator activated receptor nuclear receptors. *Prostaglandins Other Lipid Mediat.*, 62, 1-13.

EDWARDS, C. R. (1991) Lessons from licorice. *N Engl J Med,* 325**,** 1242-1243.

EDWARDS, C. R. W., BURT, D., MCINTYRE, M. A., DE KLOET, E. R., STEWART, P. M., BRETT, L., SUTANTO, W. S., & MONDER, C. (1988) Localisation of 11ß-hydroxysteroid dehydrogenase – tissue specific protector of the mineralocorticoid receptor. *The Lancet*, 332, 986-989.

EDWARDS, P.A., TABOR, D., KAST, H.R. & VENKATESWARAN, A. (2000) Regulation of gene expression by SREBP and SCAP. *Biochim Biophys Acta.*, 1529, 103-113.

EILAT-ADAR, S.[1], METE, M., FRETTS, A., FABSITZ, R.R., HANDELAND, V., LEE, E.T., LORIA, C., XU, J., YEH, J. & HOWARD, B.V. (2013) Dietary patterns and their association with cardiovascular risk factors in a population undergoing lifestyle changes: The Strong Heart Study. *Nutr Metab Cardiovasc Dis.*, 23, 528-535.

ESCHER, G., NAWROCKI, A., STAUB, T., VISHWANATH, B. S., FREY, B. M., REICHEN, J. & FREY, F. J. (1998) Down-regulation of hepatic and renal 11 beta-hydroxysteroid dehydrogenase in rats with liver cirrhosis. *Gastroenterology,* 114, 175–184.

FAN, Y., MENON, R. K., COHEN, P., HWANG, D., CLEMENS, T., DIGIROLAMO, D. J., KOPCHICK, J. J., LE ROITH, D., TRUCCO, M. & SPERLING M. A. (2009) Liver-specific deletion of the growth hormone receptor reveals essential role of growth hormone signaling in hepatic lipid metabolism. *J Biol Chem*. 284 (30), 19937-44.

FEIG P. U., SHAH S., HERMANOWSKI-VOSATKA A., PLOTKIN D., SPRINGER M. S., DONAHUE S., THACH C., KLEIN E. J., LAI E. & KAUFMAN K. D. (2011) Effects of an 11 beta-hydroxysteroid dehydrogenase type 1 inhibitor, MK-0916, in patients with type 2 diabetes mellitus and metabolic syndrome. *Diabetes Obesity Metab.,* 13, 498–504.

FERDINANDUSSE, S. & HOUTEN, S.M. (2006) Peroxisomes and bile acid synthesis. *Biochim Biophys Acta*., 1763, 1427–1440.

FEREZOU, J., COSTE, T. & CHEVALLIER, F. (1981) Origins of neutral sterols in human feces studied by stable isotope labeling (D and 13C). Existence of an external secretion of cholesterol. *Digestion*, 21, 232-243.

FILLING, C., BERNDT, K.D., BENACH, J., KNAPP, S., PROZOROVSKI, T., NORDLING, E., LADENSTEIN, R., JORNVALL, H., & OPPERMANN, U. (2002) Critical Residues for Structure and Catalysis in Short-chain Dehydrogenases/Reductases. *J Biol Chem.*, 277, 25677-25684.

FORMAN, B. M., RUAN, B., CHEN, J., SCHROEPFER, JR, G. J. & EVANS, R. M. (1997) The orphan nuclear receptor LXRa is positively and negatively regulated by distinct products of mevalonate metabolism. *Proc. Natl. Acad. Sci. USA.*, 94, 10588–10593.

FU, X., MENKE, J. G., CHEN, Y., ZHOU, G., MACNAUL, K. L., WRIGHT, S. D., SPARROW, C. P. & LUND, E. G. (2001) 27-hydroxycholesterol is an endogenous

ligand for liver X receptor in cholesterol-loaded cells. *J Biol Chem.*, 276, 38378–38387.

FUNDER, J. W. (1997) Glucocorticoid and mineralocorticoid receptors: biology and clinical relevance. *Annu Rev Med.,* 48, 231–240.

GARCÍA R. A., SEARCH D. J., LUPISELLA J. A., OSTROWSKI J., GUAN B., CHEN J., YANG W. P., TRUONG A., HE A., ZHANG R., YAN M., HELLINGS S. E., GARGALOVIC P. S., RYAN C. S., WATSON L. M., LANGISH R. A., SHIPKOVA P. A., CARSON N. L., TAYLOR J. R., YANG R., PSALTIS G. C., HARRITY T. W., ROBL J. A. & GORDON D. A. (2013) 11beta-hydroxysteroid dehydrogenase type 1 gene knockout attenuates atherosclerosis and in vivo foam cell formation in hyperlipidemic apoE$^{-/-}$ mice. *PLoS One* 8(2): e53192.

GAYLOR, J.L. (2002) Membrane-bound enzymes of cholesterol synthesis from lanosterol. *Biochem Biophys Res Commun.*, 292, 1139–1146.

GEYEREGGER, R., ZEYDA, M. & STULNIG, T.M. (2006) Liver X receptors in cardiovascular and metabolic disease. *Cell Mol Life Sci.*, 63, 524-539.

GIELEN, J. E., van CANTFORT, J. & KREMERS, P. (1976) Genetic and hormonal regulation of steroid hydroxylases and drug metabolizing enzymes in rat liver. *Arch. Toxicol.*, 36, 255–266.

GINSBERG, H. N. (1998) Lipoprotein physiology, Review. *Endocrinol Metab Clin North Am*., 27, 503-519.

GINSBERG, H.N.[1], KARMALLY, W., SIDDIQUI, M., HOLLERAN, S., TALL, A.R., RUMSEY, S.C., DECKELBAUM, R.J., BLANER, W.S. & RAMAKRISHNAN, R. (1994) A dose-response study of the effects of dietary cholesterol on fasting and postprandial lipid and lipoprotein metabolism in healthy young men. *Arterioscler Thromb.*, 14, 576-586.

GITAU, R., CAMERON, A., FISK, N. M. & GLOVER, V. (1998) Fetal exposure to maternal cortisol. *Lancet,* 352, 707-8.

GLOMSET, J.A. (1968) The plasma lecithin: cholesterol acyltransferase reaction. *J Lipid Res.*, 9, 155–167.

GLOMSET, J.A. & WRIGHT, J.L. (1964) Some properties of a cholesterol esterifying enzyme in human plasma. *Biochim Biophys Acta.*, 89, 266–276.

GNERRE, C., SCHUSTER, G.U., ROTH, A., HANDSCHIN, C., JOHANSSON, L., LOOSER, R., PARINI, P., PODVINEC, M., ROBERTSSON, K., GUSTAFSSON, J.A. & MEYER, U.A. (2005) LXR deficiency and cholesterol feeding affect the expression and phenobarbital-mediated induction of cytochromes P450 in mouse liver. *Journal of Lipid Research.*, 46, 1633–1642.

GOEDECKE J. H., WAKE D. J., LEVITT N. S., LAMBERT E. V., COLLINS M. R., MORTON N. M., ANDREW R., SECKL J. R. & WALKER B. R. (2006)

Glucocorticoid metabolism within superficial subcutaneous rather than visceral adipose tissue is associated with features of the metabolic syndrome in South African women. *Clin Endocrinol.,* 65, 81–87.

GOLDSTEIN, J.L. & BROWN, M.S. (1990) Regulation of the mevalonate pathway. *Nature*, 343, 425-430.

GOSLING, P. (1995) Albumin and the critically ill. *Care Crit Ill*, 11, 57–61.

GOULD R.G. (1951) Lipid metabolism and atherosclerosis. *Am J Med*., 11, 209-227.

GOULD, R. G., & TAYLOR, C. B. (1950) Effect of dietary cholesterol on hepatic cholesterol biosynthesis. *Fed. Proc.* 9, 179.

GOUT, J., TIRARD, J., THEVENON, C., RIOU, J. P., BEGEOT, M. & NAVILLE, D. (2006) CCAAT/enhancer-binding proteins (C/EBPs) regulate the basal and cAMP-induced transcription of the human 11β-hydroxysteroid dehydrogenase encoding gene in adipose cells. *Biochimie* 88, 1115–1124.

GRAF, G.A., YU, L., LI, W.P., GERARD, R., TUMA, P.L., COHEN, J.C. & HOBBS, H.H. (2003) ABCG5 and ABCG8 are obligate heterodimers for protein trafficking and biliary cholesterol excretion. *J Biol Chem.*, 278, 48275–48282.

GRÜNHAGE, F., ACALOVSCH, M., TIRZIU, S., WALIER, M., WIENKER, T. F., CIOCAN, A., MOSTEANU, O., SAUERBRUCH, T., LAMMERT, F. (2007) Increased gallstone risk in humans conferred by common variant of hepatic ATP-binding cassette transporter for cholesterol. *Hepatology*, 46:793–801.

GU, J., WENG, Y., ZHANG QY, CUI H, BEHR M, WU L, YANG W, ZHANG L. & DING X. (2003) Liver-specific deletion of the NADPH-cytochrome P450 reductase gene: impact on plasma cholesterol homeostasis and the function and regulation of microsomal cytochrome P450 and heme oxygenase. *J Biol Chem.,* 278 (28), 25895-901.

GUYTON J. R. & KLEMP K. F. (1996) Development of the lipid-rich core in human atherosclerosis. *Arterioscler Thromb Vasc Biol.,* 16, 4-11.

HADOKE, P., IQBAL, J., & WALKER, B. (2009). Therapeutic manipulation of glucocorticoid metabolism in cardiovascular disease. *Br J Pharmacol, 15*6, 689-712.

HALBERG N., KHAN T., TRUJILLO M. E., WERNSTEDT-ASTERHOLM I., ATTIE A. D., SHERWANI S., WANG Z. V., LANDSKRONER-EIGER S., DINEEN S., MAGALANG U. J., BREKKEN R. A. & SCHERER P. E. (2009) Hypoxia-inducible factor 1α induces fibrosis and insulin resistance in white adipose tissue. *Mol Cell Biol*, 29, 4467–4483.

HAMILTON-CRAIG I. (2001) Statin-associated myopathy. *Med J Aust*., 175, 486–489.

HAMPTON, R.Y. (2000) Cholesterol homeostasis: ESCAPe from the ER. *Curt Biol.*, 10, R298-R301.

HANNAH V. C., OU J., LUONG A., GOLDSTEIN J. L. & BROWN M. S. (2001) Unsaturated fatty acids down-regulate SREBP isoforms 1a and 1c by two mechanisms in HEK-293 cells. *J Biol Chem*., 276, 4365–4372.

HANNAH, V.C., OU, J., LUONG, A., GOLDSTEIN, J.L. & BROWN, M.S. (2001) Unsaturated fatty acids down-regulate SREBP isoforms 1a and 1c by two mechanisms in HEK-293 cells. *J Biol Chem.*, 276, 4365–4372.

HANSON, R. W. & RESHEF, L. (1997) Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. *Annu Rev Biochem,* 66**,** 581-611.

HARA, M., MATSUSHIMA, T., SATOH, H., ISO-O, N., NOTO, H., TOGO, M., KIMURA, S., HASHIMOTO, Y. & TSUKAMOTO, K. (2003) Isoform-dependent cholesterol efflux from macrophages by apolipoprotein E is modulated by cell surface proteoglycans. *Arterioscler Thromb Vasc Biol.*, 23, 269-74.

HARNO E, COTTRELL EC, KEEVIL BG, DESCHOOLMEESTER J, BOHLOOLY-Y M, ANDERSÉN H, TURNBULL AV, LEIGHTON B, & WHITE A. (2013) 11-Dehydrocorticosterone causes metabolic syndrome, which is prevented when 11β-HSD1 is knocked out in livers of male mice. Endocrinology. 154 (10), 3599-609.

HAZARD, S.E. & PATEL, S.B. (2007) Sterolins ABCG5 and ABCG8: regulators of whole body dietary sterols. *Pflugers Arch.*, 453, 745–752.

HERMANOWSKI-VOSATKA A., BALKOVEC J.M., CHENG K., CHEN H.Y., HERNANDEZ M. & KOO G.C. (2005) 11β-HSD1 inhibition ameliorates metabolic syndrome and prevents progression of atherosclerosis in mice. *J Exp Med*., 202, 517–527.

HERNELL, O., STAGGERS, J.E. & CAREY, M.C. (1990) Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 2. Phase analysis and aggregation states of luminal lipids during duodenal fat digestion in healthy adult human beings. *Biochemistry*, 29, 2041–2056.

HERSBERGER, M. & VON ECKARDSTEIN, A. (2003) Low high-density lipoprotein cholesterol: physiological background, clinical importance and drug treatment. *Drugs*, 63, 1907-1945.

HODIS H. N., CRAWFORD D. W., SEVANIAN A., AMARTEY J. K., WICKHAM E., SHARMA R. C. & BLANKENHORN D. H. (1991) Cholesterol feeding increases plasma and aortic tissue cholesterol oxide levels in parallel: further evidence for the role of cholesterol oxidation in atherosclerosis. *Atherosclerosis*, 89, 117–126.

HOLT, P.R., FAIRCHILD, B.M. & WEISS, J. (1986) A liquid crystalline phase in human intestinal contents during fat digestion. *Lipids*, 21, 444–446.

HONG C. & TONTONOZ P. (2014) Liver X receptors in lipid metabolism: opportunities for drug discovery. *Nature Reviews Drug Discovery,* 13, 433–444.

HONG C., BRADLEY M. N., RONG X., WANG X., WAGNER A., GRIJALVA V., CASTELLANI L. W., SALAZAR J., REALEGENO S., BOYADJIAN R., FOGELMAN A. M., VAN LENTEN B. J., REDDY S. T., LUSIS A. J., TANGIRALA R. K. & TONTONOZ P. (2012) LXRα is uniquely required for maximal reverse cholesterol transport and atheroprotection in ApoE-deficient mice. *Lipid Res*., 53 (6), 1126–1133.

HORNER, H. C., MUNCK, A. & LIENHARD, G. E. (1987) Dexamethasone causes translocation of glucose transporters from the plasma membrane to an intracellular site in human fibroblasts. *J Biol Chem,* 262**,** 17696-702.
HORTON J. D., GOLDSTEIN J. L. & BROWN M. S. (2002) SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest.*, 109, 1125-1131.

HORTON, J.D. & SHIMOMURA, I. (1999) Sterol regulatory element-binding proteins: activators of cholesterol and fatty acid biosynthesis. *Curr Opin Lipidol.*, 10, 143-150.

HORTON, J.D., SHIMOMURA, I., BROWN, M.S., HAMMER, R.E., GOLDSTEIN, J.L. & SHIMANO, H. (1998) Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. *J Clin Invest.*, 101, 2331–2339.

HOSOGAI, N., A. FUKUHARA, K. OSHIMA, Y. MIYATA, S. TANAKA, K. SEGAWA, S. FURUKAWA, Y. TOCHINO, R. KOMURO, M. MATSUDA, & I. SHIMOMURA. (2007) Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes,* 56, 901-911.

HSU H. J., HSU N. C., HU M. C. & CHUNG B. C. (2006) Steroidogenesis in zebrafish and mouse models. *Mol Cell Endocrinol*., 248, 160-163.

HUA X., YOKOYAMA C., WU J., BRIGGS M. R., BROWN M. S., GOLDSTEIN J. L. & WANG X. (1993) SREBP-2, a second basic-helix-loop-helix-leucine zipper protein that stimulates transcription by binding to a sterol regulatory element. *Proc Natl Acad Sci USA.*, 90 (24), 11603–11607.

HUA, X., SAKAI, J., BROWN, M. S. & GOLDSTEIN J. L. (1996) Regulated cleavage of sterol regulatory element binding proteins requires sequences on both sides of the endoplasmic reticulum membrane. *J. Biol. Chem.*, 271, 10379–10384.

HUANG Y., LIU X. Q., RALL S. C. J., TAYLOR J. M., VON ECKARDSTEIN A., ASSMANN G. & MAHLEY R. W. (1998) Overexpression and accumulation of apolipoprotein E as a cause of hypertriglyceridemia. *J. Biol. Chem*., 273, 26388–26393.

HUFF, M.W., POLLEX, R.L. & HEGELE, R.A. (2006) NPC1L1: evolution from pharmacological target to physiological sterol transporter. *Arterioscler Thromb Vasc Biol.*, 26, 2433–2438.

HUGHES H., MATHEWS B., LENZ M. L. & GUYTON J. R. (1994) Cytotoxicity of oxidized Ldl to porcine aortic smooth-muscle cells is associated with the oxysterols 7-ketocholesterol and 7-hydroxycholesterol. *Arterioscler Thromb.*, 14, 1177-1185.

HULT, M., ELLEBY, B., SHAFQAT, N., SVENSSON, S., RANE, A., JÎRNVALL, H., ABRAHMSEN, L., & OPPERMANN, U. (2004). Human and rodent type 1 11ß-hydroxysteroid dehydrogenases are 7ß-hydroxycholesterol dehydrogenases involved in oxysterol metabolism. *Cell Mol Life Sci.*, 61, 992-999.

IGNATOVA, I. D., KOSTADINOVA, R. M., GOLDRING, C. E., NAWROCKI, A. R., FREY, F. J, FREY, B. M. (2009) Tumor necrosis factor-α upregulates 11β-hydroxysteroid dehydrogenase type 1 expression by CCAAT/enhancer binding protein-β in HepG2 cells. *Am J Physiol Endocrinol Metab.,* 296: E367–E377.

IM, S. S. & OSBORNE, T. F. (2011) Liver x receptors in atherosclerosis and inflammation. *Circ. Res*., 108 (8), 996-1001.

IREDALE, J.P. (2007) Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. *J Clin Invest* 117 (3), 539-548.

ISHIBASHI, S., GOLDSTEIN, J.L., BROWN, M.S., HERZ, J. & BURNS, D.K. (1994) Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. *J Clin Invest.*, 93, 1885-1893.

ISTVAN, E.S. & DEISENHOFER, L. (2001) Structural mechanism for statin inhibition of HMGCoA reductase. *Science*, 232, 1160–1164.

IYER, S.P., YAO, X., CRONA, J.H., HOOS, L.M., TETZLOFF, G., DAVIS, H.R. JR, GRAZIANO, M.P. & ALTMANN, S.W. (2005) Characterization of the putative native and recombinant rat sterol transporter Niemann-Pick C1 Like 1 (NPC1L1) protein. *Biochim Biophys Acta.*, 1722, 282–292.

JAMIESON, P. M., CHAPMAN, K. E., EDWARDS, C. R. & SECKL J. R. (1995) 11 beta-hydroxysteroid dehydrogenase is an exclusive 11 beta- reductase in primary cultures of rat hepatocytes: effect of physicochemical and hormonal manipulations. *Endocrinology*, 136 (11), 4754-61.

JANOWSKI B. A., WILLY P. J., DEVI T. R., FALCK J. R. & MANGELSDORF D. J. (1996) An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature*, 383, 728–731.

JANOWSKI, B. A., SHAN, B. & RUSSELL, D. W. (2001) The hypocholesterolemic agent LY295427 reverses suppression of sterol regulatory element-binding protein processing mediated by oxysterols. *J. Biol. Chem*., 276, 45408–45416.

JANOWSKI, B.A., GROGAN, M.J., JONES, S.A., WISELY, G.B., KLIEWER, S.A., COREY, E.J. & MANGELSDORF, D.J. (1999) Structural requirements of ligands for the oxysterol liver X receptors LXRα and LXRβ. *Proc Natl Acad Sci U S A.,* 96, 266–271.

JAVITT, N.B. (1994) Bile acid synthesis from cholesterol: regulatory and auxillary pathways. *FASEB J*, 8, 1308-1311.

JAVITT, N.B., PFEFFER, R., KOK, E., BURSTEIN, S., COHEN, B.I. & BUDAI, K. (1989) Bile acid synthesis in cell culture. *J boil Chem.*, 264, 10384-10387.

JENSEN, K.K., PREVIS, S.F., ZHU, L., HERATH, K., WANG, S.P., BHAT, G., HU, G., MILLER, P.L., MCLAREN, D.G., SHIN, M.K., VOGT, T.F., WANG, L., WONG, K.K., RODDY, T.P., JOHNS, D.G. & HUBBARD, B.K. (2012) Demonstration of diet-induced decoupling of fatty acid and cholesterol synthesis by combining gene expression array and 2H2O quantification. *Am J Physiol Endocrinol Metab.*, 15, 302.

JESSUP, W. & KRITHARIDES, L. (2008) Lipid metabolism: recent progress in defining the contributions of cholesterol transporters to cholesterol efflux in vitro and in vivo. *Current Opinion in Lipidology*, 19 (2), 212-214.

JIANG G., MCKENZIE T. L., CONRAD D. G., SHECHTER I. (1993) Transcriptional regulation by lovastatin and 25-hydroxycholesterol in HepG2 cells and molecular cloning and expression of the cDNA for the human hepatic squalene synthase. *J Biol Chem*., 268:12818–12824.

JOHANSSON A., ANDREW R., FORSBERG H., CEDERQUIST K., WALKER B. R. & OLSSON T. (2001) Glucocorticoid metabolism and adrenocortical reactivity to ACTH in myotonic dystrophy. *J Clin Endocrinol Metab.,* 86 (9), 4276-83.

JOSEPH, S. B., CASTRILLO, A., LAFFITTE, B. A., MANGELSDORF, D. J. & TONTONOZ P. (2003) Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nat Med*., 9, 213–219.

JOSEPH, S.B., LAFFITTE, B.A., PATEL, P.H., WATSON, M.A., MATSUKUMA, K.E., WALCZAK, R., COLLINS, J.L., OSBORNE, T.F. & TONTONOZ, P. (2002b) Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors. *J Biol Chem.*, 277, 11019–11025.

JOSEPH, S.B., MCKILLIGIN, E., PEI, L., WATSON, M.A., COLLINS, A.R., LAFFITTE, B.A., CHEN, M., NOH, G., GOODMAN, J., HAGGER, G.N., TRAN, J., TIPPIN, T.K., WANG, X., LUSIS, A.J., HSUEH, W.A., LAW, R.E., COLLINS, J.L., WILLSON, T.M. & TONTONOZ, P. (2002a) Synthetic LXR ligand inhibits the development of atherosclerosis in mice. *Proc Natl Acad Sci U S A.*, 99, 7604–7609.

KALAANY, N.Y., GAUTHIER, K.C., ZAVACKI, A.M., MAMMEN, P.P., KITAZUME, T., PETERSON, J.A., HORTON, J.D., GARRY, D.J., BIANCO, A.C. & MANGELSDORF, D.J. (2005) LXRs regulate the balance between fat storage and

oxidation. *Cell Metab.*, 1, 231-244.

KANDUTSCH, A. A., & PACKIE, R.M. (1970) Comparison of the effects of some C27-, C21-, and C19-steroids upon hepatic sterol synthesis and hydroxymethylglutaryl-CoA reductase activity. *Arch Biochem Biophys.*, 140, 122-130.

KANG M. H., ZHANG L. H., WIJESEKARA N., DE HAAN W., BUTLAND S., BHATTACHARJEE A. & HAYDEN M. R. (2013) Regulation of ABCA1 protein expression and function in hepatic and pancreatic islet cells by miR-145 *Arterioscler Thromb Vasc Biol.,* 33 (12), 2724-32.

KANNISTO K., PIETILÄINEN K. H., EHRENBORG E., RISSANEN A., KAPRIO J., HAMSTEN A. & YKI-JÄRVINEN H. (2004) Overexpression of 11beta-hydroxysteroid dehydrogenase-1 in adipose tissue is associated with acquired obesity and features of insulin resistance: studies in young adult monozygotic twins. *J Clin Endocrinol Metab*., 89 (9), 4414-21.

KAREN, S.M. & ERAN, L. (2004) Atherosclerosis in the apolipoprotein e–deficient mouse, A decade of progress. *Arteriosclerosis, Thrombosis, and Vascular Biology.,* 24, 1006-1014.

KATSIKA, D., MAGNUSSON, P., KRAWCZYK, M., GRÜNHAGE, F., LICHTENSTEIN, P., EINARSSON, C., LAMMERT, F. & MARSCHALL, H.U. (2010) Gallstone disease in Swedish twins: risk is associated with ABCG8 D19H genotype. *J Intern Med.*, 268, 279–285.

KEES-FOLTS D. & DIAMOND J. R. (1993) Relationship between hyperlipidemia, lipid mediators, and progressive glomerulosclerosis in the nephrotic syndrome. *Am J Nephrol.,* 13, 365-375.

KELLEY, R.I. & HERMAN, G.E. (2001) Inborn errors of sterol biosynthesis. *A Rev Genomics Hum Genet.*, 2, 299–341.

KENNEDY, M.A., BARRERA, G.C., NAKAMURA, K., BALDÁN, A., TARR, P., FISHBEIN, M.C., FRANK, J., FRANCONE, O.L. & EDWARDS, P.A. (2005) ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. *Cell Metab.*, 1, 121– 131.

KENNEDY, M.A., VENKATESWARAN, A., TAR,R P.T., XENARIOS, I., KUDOH, J., SHIMIZU, N. & EDWARDS, P.A. (2001) Characterization of the human ABCG1 gene. Liver X receptor activates an internal promoter that produces a novel transcript encoding an alternative form of the protein. *J Biol Chem.*, 276, 39438–39447.

KERN F. Jr. (1991) Normal plasma cholesterol in an 88-year-old man who eats 25 eggs a day - mechanisms of adaptation. *N Engl J Med*., 324, 896-899.

KIM, H.J., TAKAHASHI, M. & EZAKI, O. (1999) Fish oil feeding decreases mature sterol regulatory element-binding protein 1 (SREBP-1) by down-regulation of SREBP-1c mRNA in mouse liver. A possible mechanism for down-regulation of lipogenic enzyme mRNAs. *J Biol Chem.*, 274, 25892–25898.

KINGSBURY, K. & BONDY, G. (2007) Understanding the essentials of blood lipid metabolism. *Prog Cardiovasc Nurs.*, 18, 13-18.

KIPARI T., HADOKE P. W., IQBAL J., MAN T. Y., MILLER E., COUTINHO A. E., ZHANG Z., SULLIVAN K. M., MITIC T., LIVINGSTONE D. E., SCHRECKER C., SAMUEL K., WHITE C. I., BOUHLEL M. A., CHINETTI-GBAGUIDI G., STAELS B., ANDREW R., WALKER B. R., SAVILL J. S., CHAPMAN K. E. & SECKL J. R. (2013) 11β-hydroxysteroid dehydrogenase type 1 deficiency in bone marrow-derived cells reduces atherosclerosis. *FASEB J.*, 27 (4), 1519–1531.

KLETT E. L., LEE M. H., ADAMS D. B., CHAVIN K. D. & PATEL S. B. (2004b) Localization of ABCG5 and ABCG8 proteins in human liver, gall bladder and intestine. *BMC Gastroenterol*., 4:21.

KLETT E. L., LU K., KOSTERS A., VINK E., LEE M. H., ALTENBURG M., SHEFER S., BATTA A. K., YU H., CHEN J., KLEIN R., LOOIJE N., OUDE-ELFERINK R., GROEN A. K., MAEDA N., SALEN G. & PATEL S. B. (2004a) A mouse model of sitosterolemia: absence of Abcg8/sterolin–2 results in failure to secrete biliary cholesterol. *BMC Med*., 2:5.

KOTELEVTSEV Y., HOLMES M. C., BURCHELL A., HOUSTON P. M., SCHMOLL D., JAMIESON P., BEST R., BROWN R., EDWARDS C. R., SECKL J. R. & MULLINS J. J. (1997) 11β-hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress. *Proc Natl Acad Sci. USA,* 94, 14924–14929.

KOTOKORPI, P., ELLIS, E., PARINI, P., NILSSON, L.M., STROM, S., STEFFENSEN, K.R., GUSTAFSSON, J.A. & MODE, A. (2007) Physiological differences between human and rat primary hepatocytes in response to liver X receptor activation by 3-[3-[N-(2-chloro-3-trifluoromethylbenzyl)-(2,2-diphenylethyl)amino]propyloxy]phenylacetic acid hydrochloride (GW3965).*Mol Pharmacol*, 72, 947-955.

KOVACS, W.J., OLIVIER, L.M. & KRISANS, S.K. (*2002*) Central role of peroxisomes in isoprenoid biosynthesis. *Prog Lipid Res.*, *41*, *369*–391.

KROMHOUT D. (1999) Serum cholesterol in cross-cultural perspective. The Seven Countries Study. *Acta Cardiol*., 54 (3), 155-8.

KROZOWSKI, Z., STUCHBERRY, S., WHITE, P. C., MONDER, C., & FUNDER, J. W. (1990) Characterisation of 11ß-hydroxysteroid dehydrogenase gene expression: identification of multiple unique forms of messenger ribonucleic acid in the rat kidney. *Endocrinology*, 127, 3009-3013.

KURANO, M., ISO-O, N., HARA, M., ISHIZAKA, N., MORIYA, K., KOIKE, K. & TSUKAMOTO, K. (2011) LXR agonist increases apoE secretion from HepG2 spheroid, together with an increased production of VLDL and apoE-rich large HDL. *Lipids in Health and Disease*, 10:134.

KUSHWAHA, R.S., BARRETT, P.H., REARDON, CA., LEWIS, D.S., CAREY, K.D., GETZ, G.S. & MCGILL, H.C. JR. (1995) Relationships of plasma and hepatic variables with rates of plasma low-density lipoprotein apolipoprotein B metabolism in baboons fed low- and high-fat diets. *Metabolism,* 44, 1058-1066.http://www.ncbi.nlm.nih.gov/pubmed/7637648

LAFFITTE, B.A., REPA, J.J., JOSEPH, S.B., WILPITZ, D.C., KAST, H.R., MANGELSDORF, D.J. & TONTONOZ, P. (2001) LXRs control lipid-inducible expression of the apolipoprotein E gene in macrophages and adipocytes. *Proc. Natl. Acad. Sci. USA*, 98, 507-512.

LANGDON, R.G. & BLOCH, K. (1953) The effect of some dietary additions on the synthesis of cholesterol from acetate in vitro. *J. Biol. Chem.*, 202, 77-81.

LANGER, C., HUANG, Y., CULLEN, P., WIESENHUTTER, B., MAHLEY, R.W., ASSMANN, G. & von ECKARDSTEIN, A. (2000) Endogenous apolipoprotein E modulates cholesterol efflux and cholesteryl ester hydrolysis mediated by high-density lipoprotein-3 and lipid-free apolipoproteins in mouse peritoneal macrophages. *J Mol Med.*, 78, 217–227.

LARREDE, S., QUINN, C.M., JESSUP, W., FRISDAL, E., OLIVIER, M., HSIEH, V., KIM, M-J., VAN ECK, M., COUVERT, P., CARRIE, A., GIRAL, P., CHAPMAN, M.J., GUERIN, M. & LE GOFF, W. (2009) Stimulation of cholesterol efflux by LXR agonists in cholesterol-loaded human macrophages is ABCA1-dependent but ABCG1-independent. *Arterioscler Thromb Vasc Biol.*, 29, 1930–1936.

Larsson D. A., Baird S., Nyhalah J. D., Yuan X. M. & Li W. (2006). Oxysterol mixtures, in atheroma-relevant proportions, display synergistic and proapoptotic effects. *Free Radic Biol Med.,* 41, 902-910.

LAVERY GG, ZIELINSKA AE, GATHERCOLE LL, HUGHES B, SEMJONOUS N, GUEST P, SAQIB K, SHERLOCK M, REYNOLDS G, MORGAN SA, TOMLINSON JW, WALKER EA, RABBITT EH & STEWART PM. (2012) Lack of significant metabolic abnormalities in mice with liver-specific disruption of 11β-hydroxysteroid dehydrogenase type 1. *Endocrinology*, 153 (7), 3236-48.

LEE, M.H., LU, K., HAZARD, S., YU, H., SHULENIN, S., HIDAKA, H., KOJIMA, H., ALLIKMETS, R., SAKUMA, N., PEGORARO, R., SRIVASTAVA, A.K., SALEN, G., DEAN, M. & PATEL, S.B. (2001) Identification of a gene, ABCG5, important in the regulation of dietary cholesterol absorption. *Nat Genet.*, 27, 79–83.

LEFEBVRE, M., LOVEJOY, J. & SMITH, S. (1999) Acute effects of dietary trans fatty acids on postprandial insulin, glucose and triglyceride levels. *FASEB J.*, 13, A54.

LEHMANN, J.M., KLIEWER, S.A., MOORE, L.B., SMITH-OLIVER, T.A., OLIVER, B.B., SU, J.L., SUNDSETH, S.S., WINEGAR, D.A., BLANCHARD, D.E., SPENCER, T.A. & WILLSON, T.M. (1997) Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. *J Biol Chem*., 272, 3137–3140.

LEMBERGER, T., STAELS, B., SALADIN, R., DESVERGNE, B., AUWERX, J., & WAHLI, W. (1994) Regulation of the peroxisome proliferator-activated receptor alpha gene by glucocorticoids. *J Biol Chem*., 269 (40), 24527-30.

LEWIS, G.F. (2006) Determinants of plasma HDL concentrations and reverse cholesterol transport. *Curr Opin Cardiol.*, 21, 345–352.

LI-HAWKINS J., LUND E. G., TURLEY S. D. & RUSSELL D. W. (2000) Disruption of the oxysterol 7α-hydroxylase gene in mice. *J Biol Chem.,* 275, 16536–16542.

LI, A.C. & GLASS, C.K. (2004) PPAR- and LXR-dependent pathways controlling lipid metabolism and the development of atherosclerosis. *J Lipid Res.*, 45, 2161–2173.

LI, G., HERNANDEZ-ONO, A., CROOKE, R.M., GRAHAM, M.J. & GINSBERG, H.N. (2011) Effects of antisense-mediated inhibition of 11β-hydroxysteroid dehydrogenase type 1 on hepatic lipid metabolism. *J Lipid Res.*, 52, 971–981.

LI, T., MATOZEL, M., BOEHME, S., KONG, B., NILSSON, L.-M., GUO, G., ELLIS, E. & CHIANG, J. Y. L. (2011b) Overexpression of cholesterol 7α-hydroxylase promotes hepatic bile acid synthesis and secretion and maintains cholesterol homeostasis. *Hepatology*, 53, 996–1006.

LIBBY, P. (2002) Atherosclerosis: The new view. *Scientific American*, 286, 47-55.

LIMI, T.C. (1967) The effect of cholesterol feeding and fasting upon beta-hydroxy-beta-methylglutaryl coenzyme A reductase. *J Biol Chem.*, 242, 990-993.

LIN, E.C., FERNANDEZ, M.L. & MCNAMARA, D.J. (1992) Dietary fat type and cholesterol quantity interact to affect cholesterol metabolism in guinea pigs. *J Nutr.*, 122, 2019-2029.

LIN, J., YANG, R., TARR, P.T., WU, P.H., HANDSCHIN, C., LI, S., YANG, W., PEI, L., ULDRY, M., TONTONOZ, P., NEWGARD, C.B. & SPIEGELMAN, B.M. (2005) Hyperlipidemic effects of dietary saturated fats mediated through PGC-1β coactivation of SREBP. *Cell,* 120, 261–273.

LISCUM, L. (2002) Cholesterol biosynthesis. IN: DENNIS, E.V. & JEAN, E.V. (Eds.) *New Comprehensive Biochemistry, chapter 15*, Amsterdam: Elsevier Science, pp 409-431.

LIVINGSTONE, D. E. W. & WALKER, B. R. (2003) Is 11beta-hydroxysteroid dehydrogenase type 1 a therapeutic target? Effects of carbenoxolone in lean and obese Zucker rats. *J Pharmacol Exp Therapeut*., 305:167–172.

LLOYD D. J., HELMERING J., CORDOVER D., BOWSMAN M., CHEN M., HALE C., FORDSTROM P., ZHOU M., WANG M., KAUFMAN S. A. & VENIANT M. M. (2009) Antidiabetic effects of 11β-HSD1 inhibition in a mouse model of combined diabetes, dyslipidaemia and atherosclerosis. *Diabetes Obes Metab.*, 11, 688–699.

LLOYD-JONES, D., ADAMS, R., CARNETHON, M., DE SIMONE, G., FERGUSON, T.B., FLEGAL, K., FORD, E., FURIE, K., GO, A., GREENLUND, K., HAASE, N., HAILPERN, S., HO, M., HOWARD, V., KISSELA, B., KITTNER, S., LACKLAND, D., LISABETH, L., MARELLI, A., MCDERMOTT, M., MEIGS, J., MOZAFFARIAN, D., NICHOL, G., O'DONNELL, C., ROGER, V., ROSAMOND, W., SACCO, R., SORLIE, P., STAFFORD, R., STEINBERGER, J., THOM, T., WASSERTHIEL-SMOLLER, S., WONG, N., WYLIE-ROSETT, J. & HONG, Y. (2009) Heart disease and stroke statistics--2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation.*, 119, 480–486.

LOVEJOY, J.C., CHAMPAGNE, C.M., SMITH, S.R., DELANY, J.P., BRAY, G.A., LEFEVRE, M., DENKINS, Y.M. & ROOD, J.C. (2001) Relationship of dietary fat and serum cholesterol ester and phospholipid fatty acids to markers of insulin resistance in men and women with a range of glucose tolerance. *Metabolism*, 50, 86-92.

LU, K., LEE, M.H., HAZARD, S., BROOKS-WILSON, A., HIDAKA, H., KOJIMA, H., OSE, L., STALENHOEF, A.F., MIETINNEN, T., BJORKHEM, I., BRUCKERT, E., PANDYA, A., BREWER, H.B. JR, SALEN, G., DEAN, M., SRIVASTAVA, A. & PATEL S.B. (2001) Two genes that map to the STSL locus cause sitosterolemia: genomic structure and spectrum of mutations involving sterolin-1 and sterolin-2, encoded by ABCG5 and ABCG8, respectively. *Am J Hum Genet.*, 69, 278–290.

LUND, E. G., MENKE, J. G. AND SPARROW, C. P. (2003) Liver X receptor agonists as potential therapeutic agents for dyslipidemia and atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.*, 23, 1169–1177.

LUND, E.G., PETERSON, L.B., ADAMS, A.D., LAM, M.H., BURTON, C.A., CHIN, J., GUO, Q., HUANG, S., LATHAM, M., LOPEZ, J.C., MENKE, J.G., MILOT, D.P., MITNAUL, L.J., REX-RABE, S.E., ROSA, R.L., TIAN, J.Y., WRIGHT, S.D. & SPARROW, C.P. (2006) Different roles of liver X receptor alpha and beta in lipid metabolism: Effects of an alpha-selective and a dual agonist in mice deficient in each subtype. *Biochem Pharmacol.*, 71, 453-463.

LUNDSGAARD-HANSEN, P. (1986) Physiology and pathophysiology of colloid osmotic pressure and albumin metabolism. *Curr Stud Hematol Blood Transfusion*, 53, 1–17.

LUO M. J., THIERINGER R., SPRINGER M. S., WRIGHT S. D., HERMANOWSKI-VOSATKA A. & PLUMP A. (2013) 11β-HSD1 inhibition reduces atherosclerosis in mice by altering pro-inflammatory gene expression in the vasculature. *Physiol Genomics.*, 45, 47–57.

MA AZ, SONG ZY & ZHANG Q. (2014) Cholesterol efflux is LXRα isoform-dependent in human macrophages. *BMC Cardiovasc Disord*. 14 : 80.

MACARTHUR, J.M., BISHOP, J.R., STANFORD, K.I., WANG, L., BENSADOUN, A., WITZTUM, J.L. & ESKO, J.D. (2007) Liver heparan sulfate proteoglycans mediate clearance of triglyceride-rich lipoproteins independently of LDL receptor family members. *J Clin Invest.*, 117, 153–164.

MADSEN, L., PETERSEN, R.K. & KRISTIANSEN, K. (2005) Regulation of adipocyte differentiation and function by polyunsaturated fatty acids. *Biochim Biophys Acta.*, 1740, 266-286.

MAHMOOD S. S., LEVY D., VASAN R. S. & WANG T. J. (2013) The Framingham Heart Study and the epidemiology of cardiovascular disease: a historical perspective. *Lancet* 27 (9921), 61752–3.

MARCHESINI, G., BUGIANESI, E., FORLANI, G., CERRELLI, F., LENZI, M., MANINI, R., NATALE, S., VANNI, E., VILLANOVA, N., MELCHIONDA, N. & RIZZETTO, M. (2003) Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. *Hepatology.*, 37, 917–923.

MARDONES, P., QUIÑONES, V., AMIGO, L., MORENO, M., MIQUEL, J.F., SCHWARZ, M., MIETTINEN, H.E., TRIGATTI, B., KRIEGER, M., VANPATTEN, S., COHEN, D.E. & RIGOTTI, A. (2001) Hepatic cholesterol and bile acid metabolism and intestinal cholesterol absorption in scavenger receptor class B type I-deficient mice. *J Lipid Res.*, 42, 170–180.

MARINIELLO B., RONCONI V., RILLI S., BERNANTE P., BOSCARO M., MANTERO F. & GIACCHETTI G. (2006) Adipose tissue 11beta-hydroxysteroid dehydrogenase type 1 expression in obesity and Cushing's syndrome. *Eur J Endocrinol*.,155 (3), 435-41.

MASER, E. & OPPERMANN, U. C. (1997) Role of type-1 11β-hydroxysteroid dehydrogenase in detoxification processes. *Eur J Biochem.,* 249, 365–369.

MASER E*., VOLKER B. & FRIEBERTSHAUSER J. (2002) 11β-Hydrxysteroid dehydrogenase type 1 from human liver: dimerization and enzyme cooperativity support its postulated role as glucocorticoid reductase. Biochemistry,* 41, 2459*–2465.*

MASUZAKI, H., PATERSON, J., SHINYAMA, H., MORTON, N.M., MULLINS, J.J., SECKL, J.R.& FLIER, J.S. (2001) A transgenic model of visceral obesity and the metabolic syndrome. *Science*, 294, 2166-2170.

MATER, M., THELEN, A. P., PAN, D. A. & JUMP, D. B. (1999)Sterol Response Element-binding Protein 1c (SREBP1c) Is involved in the polyunsaturated fatty acid uppression of Hepatic S14 gene transcription. *J Biol Chem.,* 274, 32725–32732.

MAXFIELD, F. R. & TABAS, I. (2005) Role of cholesterol and lipid organization in disease. *Nature,* 438, 612-621.

MAXWELL, K.N., SOCCIO, R.E., DUNCAN, E.M., SEHAYEK, E. & BRESLOW, J.L. (2003) Novel putative SREBP and LXR target genes identified by microarray analysis in liver of cholesterol-fed mice. *The Journal of Lipid Research,* 44, 2109-2119.

MAZZONE, T. (1996) Apolipoprotein E secretion by macrophages: Its potential physiological functions. *Curr Opin Lipidol.*, 7, 303–307.

MCEWEN, B. S., BIRON, C. A., BRUNSON, K. W., BULLOCH, K., CHAMBERS, W. H., DHABHAR, F. S., GOLDFARB, R. H., KITSON, R. P., MILLER, A. H., SPENCER, R. L. & WEISS, J. M. (1997) The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions. *Brain Res. Rev*., 23, 79–133.

MERCER, W., OBEYESEKERE, V., SMITH, R. & KROZOWSKI, Z. (1993) Characterization of 11β-HSD1B gene expression and enzymatic activity. *Mol Cell Endocrinol.,* 92: 247–251.

MICHAILIDOU Z, TURBAN S, MILLER E, ZOU X, SCHRADER J, RATCLIFFE PJ, HADOKE PW, WALKER BR, IREDALE JP, MORTON NM, & SECKL JR. (2012) Increased angiogenesis protects against adipose hypoxia and fibrosis in metabolic disease-resistant 11β-hydroxysteroid dehydrogenase type 1 (HSD1)-deficient mice. *J Biol Chem*., 287 (6), 4188-97.

MIKHAILIDIS D. P., LAWSON R. W., MCCORMICK A. L., SIBBRING G. C., TERSHAKOVEC A. M., DAVIES G. M. & TUNCELI K. (2011) Comparative efficacy of the addition of ezetimibe to statin vs statin titration in patients with hypercholesterolaemia: systematic review and meta-analysis. *Curr Med Res Opin*., 27, 1191-210.

MILLAR, J.S., LICHTENSTEIN, A.H., CUCHEL, M., DOLNIKOWSKI, G.G., HACHEY, D.L., COHN, J.S. & SCHAEFER, E.J. (1995) Impact of age on the metabolism of VLDL, IDL, and LDL apolipoprotein B-100 in men. *J Lipid Res.*, 36, 1155-1167.

MILLER, L. L., BLY, C. G., WATSON, M. L. & BALE, W. F. (1951) The dominant role of the liver in plasma protein synthesis. A direct study of the isolated perfused rat liver with the aid of lysine-Œ-C^{14}. *J Exp Med*., 94, 431–53.

MITIC, T., HADOKE, P. W. F., CHUAIPHICHAI, S., MAN, T. Y., MILLER, E., ANDREW, R., WALKER, B. R., CHAPMAN, K. E., & SECKL, J R. (2010) 11β-HSD1 deficiency attenuates atherosclerosis in ApoE−/− mice: role of both glucocorticoid and non-glucocorticoid (oxysterol) factors. BSCR Spring 2010 Meeting, Abstracts *Heart* 2010, 96:e9.

MITRO, N., MAK, P.A., VARGAS, L., GODIO, C., HAMPTON, E., MOLTENI, V., KREUSCH, A. & SAEZ, E. (2007) The nuclear receptor LXR is a glucose sensor. *Nature*, 445, 219-223.

MOISAN, M. P., EDWARDS, C. R. & SECKL, J. R.(1992) Differential promoter usage by the rat 11 beta-hydroxysteroid dehydrogenase gene. *Mol Endocrinol*., 6, 1082–1087.

MONDER, C. & SHACKLETON, C. H. (1984) 11 beta-Hydroxysteroid dehydrogenase: fact or fancy? *Steroids*, 44, 383-417.

MONTAGUE, C.T. & O'RAHILLY, S. (2000) The perils of portliness: causes and consequences of visceral adiposity. *Diabetes*, 49, 883-888.

MOORE, X. L., HOONG, I. & COLE, T. J. (2000) Expression of the 11beta-hydroxysteroid dehydrogenase 2 gene in the mouse. *Kidney Int.,* 57, 1307-12.

MOREAU, R. A., WHITAKER, B. D. & HICKS, K. B. (2002) Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. *Prog Lipid Res*., 41 (6), 457-500.

MORGAN, S. A., MCCABE, E. L., GATHERCOLE, L. L., HASSAN-SMITH, Z. K., LARNER, D. P., BUJALSKA, I. J., STEWART, P. M., TOMLINSON, J.W. & LAVERY, G. G. (2014) 11β-HSD1 is the major regulator of the tissue-specific effects of circulating glucocorticoid excess. *Proc Natl Acad Sci USA*., 111 (24), E2482-91.

MORGAN, S. A., SHERLOCK, M., GATHERCOLE, L. L., LAVERY, G. G., LENAGHAN, C., BUJALSKA, I. J, LABER, D., YU, A., CONVEY, G., MAYERS, R., HEGYI, K., SETHI, J. K., STEWART, P. M., SMITH, D. M. & TOMLINSON, J. W. (2009) 11beta-hydroxysteroid dehydrogenase type 1 regulates glucocorticoid-induced insulin resistance in skeletal muscle. *Diabetes*, 58 (11), 2506-2515.

MORTON N. M., HOLMES M. C., FIEVET C., STAELS B., TAILLEUX A., MULLINS J. J. & SECKL J. R. (2001) Improved lipid and lipoprotein profile, hepatic insulin sensitivity, and glucose tolerance in 11β-hydroxysteroid dehydrogenase type 1 null mice. *J Biol Chem.*, 276, 41293–41300.
MORTON N. M., PATERSON J. M., MASUZAKI H., HOLMES M. C., STAELS B., FIEVET C., WALKER B. R., FLIER J. S., MULLINS J. J. & SECKL J. R. (2004) Novel adipose tissue-mediated resistance to diet-induced visceral obesity in 11β-hydroxysteroid dehydrogenase type 1-deficient mice. *Diabetes*, 53, 931–938.

MORTON, N., PATERSON, J., MASUZAKI, H., HOLMES, M.C., STAELS, B., FIEEVET, C., WALKER, B., FLIER, J., MULLINS, J., SECKL, J.R. (2003) Reduced 11β-Hydroxysteroid dehydrogenase type 1-mediated intra-adipose glucocorticoid regeneration: a novel protective adaptation to and treatment for the metabolic syndrome. Program& Abstracts of the 85th Annual Meeting of the Endocrine Society, 2003, June 19- 22, abstract OR39-5 114.

MUNEYYIRCI-DELALE, O., LAKSHMI, V., MCCALLA, C. O., KARACAN, M., NEIL, G. & CAMILIEN, L. (1996) Variations in human placental 11 beta-dehydrogenase and 11-oxoreductase activities of 11 beta-hydroxysteroid dehydrogenase enzyme during pregnancy. *Early Pregnancy,* 2, 201-6.

MUSCAT, G.E., WAGNER, B.L., HOU, J., TANGIRALA, R.K., BISCHOFF, E.D.,

ROHDE, P., PETROWSKI, M., LI, J., SHAO, G., MACONDRAY, G. & SCHULMAN, I.G. (2002) Regulation of cholesterol homeostasis and lipid metabolism in skeletal muscle by liver X receptors. *J Biol Chem.*, 277, 40722-40728.

MUTUNGI, G., TORRES-GONZALEZ, M., MCGRANE, M.M., VOLEK, J.S. & FERNANDEZ, M.L. (2007) Carbohydrate restriction and dietary cholesterol modulate the expression of HMG-CoA reductase and the LDL receptor in mononuclear cells from adult men. *Lipids Health Dis.*, 28, 6-34.

MYANT, N.B. & MITROPOULOS, K.A. (1977) Cholesterol 7α-hydroxylase. *J Lipid Res.*, 18, 135-153.

MZIAUT, H., KORZA, G., HAND, A. R., GERARD, C. & OZOLS, J. (1999) Targeting proteins to the lumen of endoplasmic reticulum using N-terminal domains of 11beta-hydroxysteroid dehydrogenase and the 50-kDa esterase. J Biol Chem., 274, 14122–14129.

NASHEV, L. G., CHANDSAWANGBHUWANA, C., BALAZS, Z., ATANASOV, A. G., DICK, B., FREY, F. J., BAKER, M. E. & ODERMATT, A. (2007) Hexose-6-phosphate dehydrogenase modulates 11beta-hydroxysteroid dehydrogenase type 1-dependent metabolism of 7-keto- and 7beta-hydroxy-neurosteroids. *PLoS One,* 2, e561.

NESS, G.C. & CHAMBERS, C.M. (2000) Feedback and hormonal regulation of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase: the concept of cholesterol buffering capacity. *Proc Soc Exp Biol Med.,* 224, 8-19.

NESS, G.S., KELLER, R.K. & PENDLETON, L.C. (1991) Feedback regulation of hepatic 3-hydroxy-3-methylglutaryl-CoA reductase activity by dietary cholesterol is not due to altered mRNA levels. *J Bio Chem.,* 266, 14854-14857.

NIELSEN J. H., OLSEN C. E., DUEDAHL C. & SKIBSTED L. H. (1995) Isolation and quantification of cholesterol oxides in dairy products by selected ion monitoring mass spectrometry. *J Dairy Res.,* 62, 101–113.

NORLIN, M. & WIKVALL, K. (2007) Enzymes in the Conversion of Cholesterol into Bile Acids. *Curr Mol Med.*, 7, 199–218.

NUOTIO-ANTAR, A. M., HACHEY, D. L., & HASTY, A. H. (2007). Carbenoxolone treatment attenuates symptoms of metabolic syndrome and atherogenesis in obese, hyperlipidemic mice. *Am J Physiol Endocrinol Metab*., 293, E1517-1528.

ODERMATT, A., DA CUNHA, T., PENNO, C. A., CHANDSAWANGBHUWANA, C., REICHERT, C., WOLF, A., DONG, M. & BAKER, M. E. (2011) Hepatic reduction of the secondary bile acid 7-oxolithocholic acid is mediated by 11beta-hydroxysteroid dehydrogenase 1. *Biochem J*., 436, 621–629.

OHASHI, R., MU, H., WANG, X., YAO, Q. & CHEN, C. (2005) Reverse cholesterol transport and cholesterol efflux in atherosclerosis. *QJM*, 98, 845-856.

OLIVIER, L.M. & KRISANS, S.K. (2000) Peroxisomal protein targeting and identification of peroxisomal targeting signals in cholesterol biosynthetic enzymes. *Biochim Biophys Acta.,* 1529, 89-102.

OPPERMANN, U., FILLING, C., HULT, M., SHAFQAT, N., WU, X., LINDH, M., SHAFQAT, J., NORDLING, E., KALLBERG, Y., PERSSON, B., & J˚RNVALL, H. (2003). Short-chain dehydrogenases/reductases (SDR): the 2002 update. *Chemico-Biological Interactions*, 143-144, 247-253.

OSBORNE, T.E. (2000) Sterol regulatory element-binding proteins (SREBPs): key regulators of nutritional homeostasis and insulin action. *J Biol Chem.*, 275, 32379-32382.

OZOLS J. (1995) Lumenal orientation and posttranslational modifications of the liver microsomal 11β-hydroxysteroid dehydrogenase. *J Biol Chem.,* 270, 2305–2312.

PAI J. T., GURYEV O., BROWN M. S. & GOLDSTEIN J. L. (1998) Differential stimulation of cholesterol and unsaturated fatty acid biosynthesis in cells expressing individual nuclear sterol regulatory element-binding proteins. *J Biol Chem*., 273 (40), 26138-48.

PATEL, M. B., OZA, N. A., ANAND I. S., DESHPANDE S. S., PATEL, C. N. (2008) Liver X Receptor: A Novel Therapeutic Target**.** *Indian J Pharm Sci*.,70 (2), 135–144.

PATERSON J. M., HOLMES M. C., KENYON C. J., CARTER R., MULLINS J. J., & SECKL J. R. (2007) Liver-selective transgene rescue of hypothalamic-pituitary-adrenal axis dysfunction in 11β-hydroxysteroid dehydrogenase type 1-deficient mice. *Endocrinology*, 148, 961–966.

PATERSON J.M., MORTON N.M., FIEVET C., KENYON C.J., HOLMES M.C., STAELS B., SECKL J.R. & MULLINS J.J. (2004) Metabolic syndrome without obesity: hepatic overexpression of 11β-hydroxysteroid dehydrogenase type 1 in transgenic mice. *Proc Natl Acad Sci USA*, 101, 7088–7093.

PAULMYER-LACROIX O., BOULLU S., OLIVER C., ALESSI M. C. & GRINO M. (2002) Expression of the mRNA coding for 11β-hydroxysteroid dehydrogenase type 1 in adipose tissue from obese patients: an in situ hybridization study. *J Clin Endocrinol Metab.,* 87, 2701–2705.

PAULSEN S. K., PEDERSEN S. B., FISKER S. & RICHELSEN B. (2007) 11β-HSD type 1 expression in human adipose tissue: impact of gender, obesity, and fat localization. *Obesity*, 15, 1954–1960.

PEET D. J., JANOWSKI B. A. & MANGELSDORF D. J. (1998) The LXRs: a new class of oxysterol receptors. *Curr. Opin. Genet. Dev*., 8, 571–575.

PEET, D.J., TURLEY, S.D., MA, W., JANOWSKI, B.A., LOBACCARO, J.M., HAMMER, R.E. & MANGELSDORF, D.J. (1998) Cholesterol and bile acid

metabolism are impaired in mice lacking the nuclear oxysterol receptor LXRα. *Cell*, 93, 693–704.

PENNINGS M., MEURS I., YE D., OUT R., HOEKSTRA M., VAN BERKEL T. J. &VAN ECK M. (2006) Regulation of cholesterol homeostasis in macrophages and consequences for atherosclerotic lesion development. *FEBS Lett.*, 580 (23), 5588-96.

PIKULEVA I.A., BABIKER A., WATERMAN M.R. & BJORKHEM I. (1998) Activities of recombinant human cytochrome P450c27 (CYP27) which produce intermediates of alternative bile acid biosynthetic pathways. *J Biol Chem.,* 273, 18153-18160.

PLÖSCH, T., BLOKS, V.W., TERASAWA, Y., BERDY, S., SIEGLER, K., VAN DER SLUIJS, F., KEMA, I.P., GROEN, A.K., SHAN, B., KUIPERS, F. & SCHWARZ, M. (2004) Sitosterolemia in ABC-transporter G5-deficient mice is aggravated on activation of the liver-X receptor. *Gastroenterology*, 126, 290–300.

PLUMP, A.S., SCOTT, C.J. & BRESLOW, J.L. (1994) Human apolipoprotein A-I gene expression increases high density lipoprotein and suppresses atherosclerosis in the apolipoprotein E-deficient mouse. *Proc Natl Acad Sci U S A.*, 91, 9607-9611.

PLUMP, A.S., SMITH, J.D., HAYEK, T., AALTO-SETALA, K., WALSH, A., VERSTUYFT, J.G., RUBIN, E.M. & BRESLOW, J.L. (1992) Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell*, 71, 343-353.

PORTINCASA, P., MOSCHETTA, A. & PALASCIANO, G. (2006) Cholesterol gallstone disease. *Lancet.* 368, 230–239.

POSTIC C., SHIOTA M., NISWENDER K. D., JETTON T.L., CHEN Y., MOATES J. M., SHELTON K. D., LINDNER J., CHERRINGTON A. D. & MAGNUSON M. A. (1999) Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. *J Biol Chem*., 274 (1), 305-15.

PRINCEN, H. M., MEIJER, P., & HOFSTEE, B. (1989) Dexamethasone regulates bile acid synthesis in monolayer cultures of rat hepatocytes by induction of cholesterol 7 alpha-hydroxylase. *Biochem J*., 262, 341-348.

RADHAKRISHNAN A., IKEDA Y., KWON H. J., BROWN M. S. & GOLDSTEIN J. L. (2007) Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: oxysterols block transport by binding to Insig. *Proc Natl Acad Sci USA*., 104, 6511–6518.

RAJAN, V., CHAPMAN, K. E., LYONS, V., JAMIESON, P., MULLINS, J. J., EDWARDS, C. R. W. & SECKL, J. R. (1995) Cloning sequencing and tissue-distribution of mouse 11β-hydroxysteroid dehydrogenase-1 cDNA. *J Steroid Biochem Mol Biol*. 52, 141–147.

RASK E., OLSSON T., SODERBERG S., ANDREW R., LIVINGSTONE D. E., JOHNSON O. & WALKER B. R. (2001) Tissue-specific dysregulation of cortisol metabolism in human obesity. *J Clin Endocrinol Metab.,* 86, 1418–1421.

RASK E., WALKER B. R., SODERBERG S., LIVINGSTONE D. E., ELIASSON M., JOHNSON O., ANDREW R. & OLSSON T. (2002) Tissue-specific changes in peripheral cortisol metabolism in obese women: increased adipose 11β-hydroxysteroid dehydrogenase type 1 activity. *J Clin Endocrinol Metab.,* 87: 3330–3336.

REPA J. J., BERGE K. E., POMAJZL C., RICHARDSON J. A., HOBBS H. & MANGELSDORF D. J. (2002) Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors alpha and beta. *J Biol Chem.*, 277 (21), 18793–18800.

REPA J. J., LUND E. G., HORTON J. D., LEITERSDORF E., RUSSELL D. W., DIETSCHY J. M. & TURLEY S. D. (2000a) Disruption of the sterol 27-hydroxylase gene in mice results in hepatomegaly and hypertriglyceridemia. Reversal by cholic acid feeding. *J Biol Chem.,* 275*,* 39685–39692.

REPA, J. J., TURLEY, S. D., LOBACCARO, J. A., MEDINA, J., LI, L., LUSTIG, K., SHAN, B., HEYMAN, R. A., DIETSCHY, J. M. & MANGELSDORF, D. J. (2000b) Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science*, 289, 1524-1529.

REUL JM, GESING A, DROSTE S, STEC IS, WEBER A, BACHMANN C, BILANG-BLEUEL A, HOLSBOER F, LINTHORST AC. (2000) The brain mineralocorticoid receptor: greedy for ligand, mysterious in function. *European Journal of Pharmacology* 405(1-3): 235-249.

RIEMERSMA, R.A. (2002) Oxidized fat in the diet and coronary heart disease. *Lipid Technology*, 14, 125-128.

RINNINGER, .F, BRUNDERT, M., JÄCKLE, S., GALLE, P.R., BUSCH, C., IZBICKI, J.R., ROGIERS, X., HENNE-BRUNS, D., KREMER, B. & BROELSCH, C.E. (1994) Selective uptake of high-density lipoprotein-associated cholesteryl esters by human hepatocytes in primary culture. *Hepatology*, 19, 1100–1114.

ROSE, A. J., DIAZ, M. B., REIMANN, A., KLEMENT, J., WALCHER, T., KRONES-HERZIG, A., STROBEL, O., WERNER, J., PETERS, A., KLEYMAN, A., TUCKERMANN, J. P., VEGIOPOULOS, A. & HERZIG, S. (2011) Molecular control of systemic bile acid homeostasis by the liver glucocorticoid receptor. *Cell Metab.* 14, 123–130.

ROSEN H., RESHEF A., MAEDA N., LIPPOLDT A., SHPIZEN S., TRIGER L., EGGERTSEN G., BJÖRKHEM I. & LEITERSDORF E. (1998) Markedly reduced bile acid synthesis but maintained levels of cholesterol and vitamin D metabolites in mice with disrupted sterol 27-hydroxylase gene. *J Biol Chem.,* 273*,* 14805–14812.

ROSENSTOCK J., BANARER S., FONSECA V. A., INZUCCHI S. E., SUN W., YAO W., HOLLIS G., FLORES R., LEVY R., WILLIAMS W. V., SECKL J. R. & HUBER R. (2010) The 11-beta-hydroxysteroid dehydrogenase type 1 inhibitor INCB13739 improves hyperglycemia in patients with type 2 diabetes inadequately controlled by metformin monotherapy. *Diabetes Care*., 33, 1516–1522.

ROSS S. E., ERICKSON R. L., GERIN I., DEROSE, P. M., BAJNOK, L., LONGO, K. A. MISEK, D. E., KUICK, R., HANASH, S. M., ATKINS, K. B., ANDRESEN, S. M., NEBB, H. I, MADSEN, L., KRISTIANSEN, K., & MACDOUGALD, O. A. (2002) Microarray analyses during adipogenesis: understanding the effects of Wnt signaling on adipogenesis and the roles of liver X receptor alpha in adipocyte metabolism. *Mol Cell Biol*., 22, 5989–5999.

ROZMAN D. & MONOSTORY K. (2010) Perspectives of the non-statin hypolipidemic agents. *Pharmacol Ther*., 127, 19–40.

RUBIN, E.M., KRAUSS, R.M., SPANGLER, E.A., VERSTUYFT, J.G. & CLIFT, S.M. (1991) Inhibition of early atherogenesis in transgenic mice by human apolipoprotein AI. *Nature*, 353, 265–267.

RUDLING, M. (1992) Hepatic mRNA levels for the LDL receptor and HMG-CoA reductase show coordinate regulation in vivo. *J Lipid Res.*, 33, 493-501.

RUSSELL, D.W. (2003) The enzymes, regulation, and genetics of bile acid synthesis. *Annual Review of Biochemistry*, 72, 137–174.

RUSSELL, D.W. & SETCHELL, K.D.R. (1992) Bile acid biosynthesis. *Biochem.*, 31, 4737-4749.

RUST, S., ROSIER, M., FUNKE, H., REAL, J., AMOURA, Z, PIETTE, J.C., DELEUZE, J.F., BREWER, H.B., DUVERGER, N., DENÈFLE, P. & ASSMANN, G. (1999) Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. *Nat Genet.*, 22, 352–355.

SACKS, F.M., SALAZAR, J., MILLER, L., FOSTER, J.M., SUTHERLAND, M., SAMONDS, K.W., ALBERS, J.J. & KASS, E.H. (1984) Ingestion of egg raises plasma low density lipoproteins in free-living subjects. *Lancet.*, 24, 647-649.

SAI, S., ESTEVES, C. L., KELLY, V., MICHAILIDOU, Z., ANDERSON, K., COLL, A. P, NAKAGAWA, Y., OHZEKI, T., SECKL, J. R. & CHAPMAN, K. E. (2008) Glucocorticoid regulation of the promoter of 11beta-hydroxysteroid dehydrogenase type 1 is indirect and requires CCAAT/enhancer-binding protein-beta. *Mol Endocrinol.,*22, 2049–2060.

SAKAI, J. & RAWSON, R.B. (2001) The sterol regulatory element-binding protein pathway: control of lipid homeostasis through regulated intracellular transport. *Curt Opin Lipidol.*, 12, 261-266.

SANDEEP T. C., ANDREW R., HOMER N. Z., ANDREWS R. C., SMITH K. & WALKER B. R. (2005) Increased in vivo regeneration of cortisol in adipose tissue in

human obesity and effects of the 11β-hydroxysteroid dehydrogenase type 1 inhibitor carbenoxolone. *Diabetes*, 54, 872–879.

SANDEEP T. C., YAU J. L., MACLULLICH A. M., NOBLE J., DEARY I. J., WALKER B. R. & SECKL J. R. (2004) 11-Betahydroxysteroid dehydrogenase inhibition improves cognitive function in healthy elderly men and type 2 diabetics. *Proc Natl Acad Sci USA.,* 101, 6734–6739.

SAPOLSKY, R. M., KREY, L. C. & MCEWEN, B. S. (1986) The neuroendocrinology of stress and aging: the glucocorticoid cascade hypothesis. *Endocr Rev*., 7, 284-301.

SAPOLSKY, R. M., ROMERO, L. M. & MUNCK, A. U. (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews*, 2155–89.

SATHIARAJ, E., CHUTKE, M., REDDY, M.Y., PRATAP, N., RAO, P.N., REDDY, D.N. & RAGHUNATH, M. (2011) A case–control study on nutritional risk factors in non-alcoholic fatty liver disease in Indian population. *Eur J Clin Nutr.*, 65, 533–537.

SATO, R., INOUE, J., KAWABE, Y., KODAMA, T., TAKANO, T. & MAEDA, M. (1996) Sterol-dependent transcriptional regulation of sterol regulatory element-binding protein-2. *J Biol Chem.*, 271, 26461–26464.

SCHMIDT, R. J., FICORILLI, J. V., ZHANG, Y., BRAMLETT, K. S., BEYER, T. P., BORCHERT, K., DOWLESS, M. S., HOUCK, K. A., BURRIS, T. P., EACHO, P. I., LIANG, G., GUO, L. W., WILSON, W. K, MICHAEL, L. F. & CAO, G. (2006) A 15-ketosterol is a liver X receptor ligand that suppresses sterol-responsive element binding protein- 2 activity. *Journal of Lipid Research*, 47, 1037-1044.

SCHOENHEIMER, R. & BREUSCH, F. (1933) Synthesis and destruction of cholesterol in the organism. *J Biol Chem.*, 103, 439–448.

SCHROEPFER G. J. Jr. (2000) Oxysterols: Modulators of Cholesterol Metabolism and Other Processes. *Physiol Rev.,* 80 (1), 361-554.

SCHULTZ, J.R., TU, H., LUK, A., REPA, J.J., MEDINA, J.C., LI, L., SCHWENDNER, S., WANG, S., THOOLEN, M., MANGELSDORF, D.J., LUSTIG, K.D. & SHAN, B. (2000) Role of LXRs in control of lipogenesis. *Genes Dev.*, 14, 2831-2838.

SCHUSTER, G.U., PARINI, P., WANG, L., ALBERTI, S., STEFFENSEN, K.R., HANSSON, G.K., ANGELIN, B. & GUSTAFSSON, J.A. (2002) Accumulation of foam cells in liver X receptor-deficient mice. *Circulation*, 106, 1147-1153.

SCHWARTZ, K., LAWN, R.M. & WADE, D.P. (2000) ABC1 gene expression and ApoA-I-mediated cholesterol efflux are regulated by LXR. *Biochem Biophys Res Commun.*, 274, 794-802.

SCHWARZ M., RUSSELL D. W., DIETSCHY J. M. & TURLEY S. D. (1998*)* Marked reduction in bile acid synthesis in cholesterol 7α-hydroxylase deficient mice does not lead to diminished tissue cholesterol turnover or to hypercholesterolemia. *J Lipid Res.,* 39, 1833–1843.

SCHWARZ, M., RUSSELL, D.W., DIETSCHY, J.M. & TURLEY, S.D. (2001) Alternate pathways of bile acid synthesis in the cholesterol 7α-hydroxylase knockout mouse are not upregulated by either cholesterol or cholestyramine feeding. *The Journal of Lipid Research,* 42, 1594-1603.

SCHWEIZER, R.A.S., MARCEL, Z., BALAZS, Z., BERNHARD, D., & ODERMATT, A. (2004). Rapid hepatic metabolism of 7-ketocholesterol by 11ß-hydroxysteroid dehydrogenase type 1: Species-specific differences between the rat, human, and hamster enzyme. *J Biol Chem.*, 279, 18415-18424.

SCRIVER, C.R., BEAUDET, A.L., SLY, W.S. & VALLE, D. (1995) *The metabolic and molecular bases of inherited disease.* 7th ed. New York: McGraw-Hill.

SECKL J.R. & WALKER B.R. (2001). 11b-hydroxysteroid dehydrogenase type 1: a tissue-specific amplifier of glucocorticoid action. *Endocrinology*, 142, 1371-1376.

SECKL, J. R. & CHAPMAN, K. E. (1997) The 11ß-hydroxysteroid dehydrogenase system, a determinant of glucocorticoid and mineralocorticoid action. *Eur J Biochem.,* 249, 361-364.

SEHAYEK E. & HAZEN S. L. (2008) Cholesterol absorption from the intestine is a major determinant of reverse cholesterol transport from peripheral tissue macrophages. *Arterioscler Thromb Vasc Biol*., 28, 1296-7.

SETIAWAN, E., JACKSON, M. F., MACDONALD, J. F. & MATTHEWS, S. G. (2007) Effects of repeated prenatal glucocorticoid exposure on long-term potentiation in the juvenile guinea-pig hippocampus. *The Journal of Physiology*, 581: 1033–1042.
SHAPIRO, D. J., & RODWELL, V.W. (1971) Regulation of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase and cholesterol synthesis. *J Biol Chem.*, 246, 3210-3216.

SHEPHERD, J., BLAUW, G. J., MURPHY, M. B., BOLLEN, E. L., BUCKLEY, B. M., COBBE, S. M., FORD, I., GAW, A., HYLAND, M., JUKEMA, J. W., KAMPER, A. M., MACFARLANE, P. W., MEINDERS, A. E., NORRIE, J., PACKARD, C. J., PERRY, I. J., STOTT, D. J., SWEENEY, B. J., TWOMEY, C., & WESTENDORP, R. G.; PROSPER study group. PROspective Study of Pravastatin in the Elderly at Risk. (2002) Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial. *Lancet*, 360(9346):1623-30.

SHIMANO, H., HORTON, J.D., HAMMER, R.E., SHIMOMURA, I., BROWN, M.S. & GOLDSTEIN, J.L. (1996) Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. *J Clin Invest.*, 98, 1575–1584.

SHIMANO, H., HORTON, J.D., SHIMOMURA, I., HAMMER, R.E., BROWN, M.S. & GOLDSTEIN, J.L. (1997) Isoform 1c of sterol regulatory element binding protein is less active than isoform 1a in livers of transgenic mice and in cultured cells. *J Clin Invest.*, 99, 846–854.

SHIMOMURA, I., BASHMAKOV, Y., SHIMANO, H., HORTON, J.D., GOLDSTEIN, J.L. & BROWN, M.S. (1997) Cholesterol feeding reduces nuclear forms of sterol regulatory element binding proteins in hamster liver. *Proc Natl Acad Sci USA.*, 94, 12354 –12359.

SHIN, M.J., CHO, Y., MOON, J., JEON, H.J., LEE, S.M. & CHUNG, J.H. (2013) Hypocholesterolemic effect of daily fisetin supplementation in high fat fed Sprague-Dawley rats. *Food Chem Toxicol.*, 57, 84-90.

SIMMONDS W. J., HOFMANN A. F. & THEODOR E. (1967) Absorption of cholesterol from a micellular solution: intestinal perfusion studies in man. *J Clin Invest*., 46, 874–890.

SIMONS K. & IKONEN E. (2000) How cells handle cholesterol. *Science*, 290, 1721-1726.

SIPERSTEIN, M.D. (1970) Regulation of cholesterol biosynthesis in normal and malignant tissues. IN: HORECKER, B.L. & STADTMAN, E.R. (Eds.) *Current Topics in Cellular Regulation, 2*. New York, NY: Academic Press, pp65-100.

SIRI-TARINO, P. W., SUN, Q., HU, F. B. & KRAUSS, R, M. (2010) *Meta-analysis of prospective cohort studies evaluating the association of saturated fat with cardiovascular disease. Am J Clin Nutr.,* 91, 535–46.

SLAVIN, B. G., ONG, J. M. & KERN, P. A. (1994) Hormonal regulation of hormone-sensitive lipase activity and mRNA levels in isolated rat adipocytes. *J Lipid Res,* 35**,** 1535-41.

SMITH J. R., OSBORNE T. F., BROWN M. S., GOLDSTEIN J. L. & GIL G. (1988) Multiple sterol regulatory elements in promoter for hamster 3-hydroxy-3-methylglutaryl-coenzyme A synthase. *J Biol Chem*., 263, 18480–18487.

SMITH, L.C., POWNALL, H.J. & GOTTO, A.M. (1978) The plasma lipoproteins-structure and metabolism. *Annu Rev Biochem.*, 47, 751-757.

SPADY, D. K. & DIETSCHY, J. M. (1983) Sterol synthesis in vivo in 18 tissues of the squirrel monkey, guinea pig, rabbit, hamster, and rat. *J Lipid Res.*, 24, 303–315.

SPARROW, C.P., BAFFIC, J., LAM, M.H., LUND, E.G., ADAMS, A.D., FU, X., HAYES, N., JONES, A.B., MACNAUL, K.L., ONDEYKA, J., SINGH, S., WANG, J., ZHOU, G., MOLLER, D.E., WRIGHT, S.D. & MENKE, J.G. (2002) A potent synthetic LXR agonist is more effective than cholesterol loading at inducing ABCA1 mRNA and stimulating cholesterol efflux. *J Biol Chem.*, 277, 10021-10027.

STAAB, C. A., STEGK, J. P., HAENISCH, S., NEISS, E., KOBSCH, K., EBERT, B., CASCORBI, I., MASER, E. (2011) Analysis of alternative promoter usage in expression of *HSD11B1* including the development of a transcript-specific quantitative real-time PCR method. *Chem Biol Interact*., 191, 104–112.

STAMLER, J., DAVIGLUS, M. L., GARSIDE, D. B., DYER, A. R., GREENLAND, P. & NEATON, J. D. (2000) Relationship of baseline serum cholesterol levels in 3 large cohorts of younger men to long-term coronary, cardiovascular, and all-cause mortality and to longevity. *JAMA*, 284, 311–318.

STEFFEN Y., WISWEDEL I., PETER D., SCHEWE T. & SIES H. (2006). Cytotoxicity of myeloperoxidase/nitrite-oxidized low-density lipoprotein toward endothelial cells is due to a high 7 beta-hydroxycholesterol to 7-ketocholesterol ratio. *Free Radic Biol Med.*, 41, 1139-1150.

STEWART, P. M., & KROZOWSKI, Z. S. (1999) 11ß-Hydroxysteroid dehydrogenase. *Vitamins and Hormones*, 57, 249-324.

STIMSON R. H., ANDREW R., MCAVOY N. C., TRIPATHI D., HAYES P. C. & WALKER B. R. (2011) Increased whole-body and sustained liver cortisol regeneration by 11 beta-hydroxysteroid dehydrogenase type 1 in obese men with type 2 diabetes provides a target for enzyme inhibition. *Diabetes,* 60, 720–725.

SU, K., SABEVA, N.S., LIU, J., WANG, Y., BHATNAGAR, S., van der WESTHUYZEN, D.R. & GRAF, G.A. (2012) The ABCG5 ABCG8 Sterol Transporter Opposes the Development of Fatty Liver Disease and Loss of Glycemic Control Independently of Phytosterol Accumulation. *J Biol Chem.*, 17, 287(34).

SUDHOP T., LUTJOHANN D., KODAL A., IGEL M., TRIBBLE D. L., SHAH S., PEREVOZSKAYA I. & VON BERGMANN K. (2002) Inhibition of intestinal cholesterol absorption by ezetimibe in humans. *Circulation*, 106, 1943-8.

SUMIYOSHI, M., SAKANAKA, M. & KIMURA, Y. (2006) Chronic intake of high-fat and high-sucrose diets differentially affects glucose intolerance in mice. *J Nutr.*, 136, 582-587.

SWELL, L., TROUT, E.C. JR, HOPPER, J.R., FIELD, H. JR & TREADWELL, C.R. (1958) Specific function of bile salts in cholesterol absorption. *Proc Soc Exp Biol Med.*, 98, 174–176.

TABAS, I. (2010) Macrophage death and defective inflammation resolution in atherosclerosis. *Nat Rev Immunol*., 10, 36–46.

TANGIRALA, R.K., BISCHOFF, E.D., JOSEPH, S.B., WAGNER, B.L., WALCZAK, R., LAFFITTE, B.A., DAIGE, C.L., THOMAS, D., HEYMAN, R.A., MANGELSDORF, D.J., WANG, X., LUSIS, A.J., TONTONOZ, P. & SCHULMAN, I.G. (2002) Identification of macrophage liver X receptors as inhibitors of atherosclerosis. *Proc Natl Acad Sci U S A.*, 99, 11896–11901.

TANNIN, G. M., AGARWAL, A. K., MONDER, C., NEW, M. I. & WHITE, P. C. (1991) The human gene for 11 beta-hydroxysteroid dehydrogenase. Structure, tissue distribution, and chromosomal localization. *J Biol Chem*., 266, 16653–16658.

TEBOUL M., ENMARK E., LI Q., WIKSTROM A. C., PELTO-HUIKKO M. & GUSTAFSSON J. A. (1995) OR-1, a member of the nuclear receptor superfamily that interacts with the 9-cis-retinoic acid receptor. *Proc. Natl. Acad. Sci. USA*., 92, 2096–2100.

TEMEL R. E. & BROWN J. M. (2010) A new framework for reverse cholesterol transport: non-biliary contributions to reverse cholesterol transport. *World J Gastroenterol*.,16, 5946–5952.

TEMEL R. E. & BROWN J. M. (2012) Biliary and nonbiliary contributions to reverse cholesterol transport. *Curr Opin Lipidol.*, 23 (2), 85-90.

THEWKE, D. P., PANINI, S. R. & SINENSKY, M. (1998) Oleate potentiates oxysterol inhibition of transcription from sterol regulatory element-1-regulated promoters and maturation of sterol regulatory element-binding proteins. *J Biol Chem.*, 273, 21402–21407.

TIGANESCU A., TAHRANI A. A., MORGAN S. A., OTRANTO M., DESMOULIÈRE A., ABRAHAMS L., HASSAN-SMITH Z., WALKER E. A., RABBITT E. H., COOPER M. S., AMREIN K., LAVERY G. G. & STEWART P. M. (2013) 11beta-hydroxysteroid dehydrogenase blockade prevents age-induced skin structure and function defects. *J Clin Invest.*, 123 (7), 3051-3060.

TIMMINS, J.M., LEE, J.Y., BOUDYGUINA, E., KLUCKMAN, K.D., BRUNHAM, L.R., MULYA, A., GEBRE, A.K., COUTINHO, J.M., COLVIN, P.L., SMITH, T.L., HAYDEN, M.R., MAEDA, N. & PARKS, J.S. (2005) Targeted inactivation of hepatic Abca1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I. *J Clin Invest.*, 115, 1333–1342.

TOMKIN, G. M., SHEPPARD, H., & CHAIKOFF, I. L. (1953) Cholesterol synthesis by liver. III. Its regulation by ingested cholesterol. *J. Biol. Chem.*, 201, 137-141.

TOMLINSON J. W., WALKER E. A., BUJALSKA I. J., DRAPER N., LAVERY G. G., COOPER M. S, HEWISON M. & STEWART P. M. (2004) 11β-Hydroxysteroid dehydrogenase type 1: a tissue-specific regulator of glucocorticoid response. *Endocr Rev*., 25, 831–866.

TRAPANI L., SEGATTO M. & PALLOTTINI V. (2012) Regulation and deregulation of cholesterol homeostasis: The liver as a metabolic "power station". *World J Hepatol*., 4 (6), 184-90.

TSUKAMOTO, K., MAUGEAIS, C., GLICK, J. M. & RADER, D. J. (2001) Markedly increased secretion of VLDL triglycerides induced by gene transfer of apolipoprotein E isoforms in apoE-deficient mice. *J Lipid Res*., 41, 253-9.

TURBAN S., LIU X., RAMAGE L., WEBSTER S. P., WALKER B. R., DUNBAR D. R., MULLINS J. J., SECKL J. R. & MORTON N. M. (2012) Optimal elevation of β-cell 11β-hydroxysteroid dehydrogenase type 1 is a compensatory mechanism that prevents high-fat diet-induced β-cell failure. *Diabetes*, 61 (3), 642-652.

VAGUE, J. (1956) The degree of masculine differentiation of obesities: a factor determining predisposition to diabetes, atherosclerosis, gout, and uric calculous disease. *Am J Clin Nutr.*, 4, 20-34.

VAISMAN B. L., LAMBERT G., AMAR M., JOYCE C., ITO T., SHAMBUREK R. D., CAIN W. J., FRUCHART-NAJIB J., NEUFELD E. D., REMALEY A. T., BREWER H. B. Jr. & SANTAMARINA-FOJO S. (2001) ABCA1 overexpression leads to hyperalphalipoproteinemia and increased biliary cholesterol excretion in transgenic mice. *J Clin Invest*.,108 (2), 303-9.

VALLETT S. M., SANCHEZ H .B., ROSENFELD J. M. & OSBORNE T. F. (1996) A direct role for sterol regulatory element binding protein in activation of 3-hydroxy-3-methylglutaryl coenzyme A reductase gene. *J Biol Chem*., 271, 12247–12253.

VAN DER VEEN J. N., VAN DIJK T. H., VRINS C. L., VAN MEER H., HAVINGA R, BIJSTERVELD K., TIETGE U. J., GROEN A. K. & KUIPERS F. (2009) Activation of the liver X receptor stimulates trans-intestinal excretion of plasma cholesterol. *J Biol Chem*., 284, 19211–19219.

VAN DER VELDE A. E., BRUFAU G. & GROEN A. K. (2010) Transintestinal cholesterol efflux. *Curr Opin Lipidol.*, 21, 167–171.

VAN DER VELDE A. E., VRINS C. L., VAN DEN OEVER K., KUNNE C., OUDE ELFERINK R. P., KUIPERS F. & GROEN A. K. (2007) Direct intestinal cholesterol secretion contributes significantly to total fecal neutral sterol excretion in mice. *Gastroenterology*., 133, 967–975.

VAN GAAL L.F., MERTENS I.L. & DE BLOCK C.E. (2006) Mechanisms linking obesity with cardiovascular disease. *Nature*, 444 (7121), 875-880.

van REE, J.H., van den BROEK, W.J., DAHLMANS, V.E., GROOT, P.H., VIDGEON-HART, M., FRANTS, R.R., WIERINGA, B., HAVEKES, L.M. & HOFKER, M.H. (1994) Diet-induced hypercholesterolemia and atherosclerosis in heterozygous apolipoprotein E-deficient mice. *Atherosclerosis.*, 111, 25–37.

VEGIOPOULOS, A. & HERZIG, S. (2007) Glucocorticoids, metabolism and metabolic diseases. *Mol Cell Endocrinol,* 275**,** 43-61.

VENKATESWARAN A., LAFFITTE B. A., JOSEPH S. B., MAK P. A., WILPITZ D. C., EDWARDS P. A. & TONTONOZ P. (2000) Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXRa. *Proc. Natl. Acad. Sci. USA.,* 97, 12097–12102.

VINE D. F., CROFT K. D., BEILIN L. J. & MAMO, J. C. (2002) Effect of dietary cholesterol oxidation products on the plasma clearance of chylomicrons in the rat. *Lipids,* 37, 455–462.

VLAHCEVIC Z. R., STRAVITZ R. T., HEUMAN D. M., HYLEMON P. B. & PANDAK W. M. (1997) Quantitative estimations of the contribution of different bile acid pathways to total bile acid synthesis in the rat. *Gastroenterology,* 113, 1949–1957.

VLAHCEVIC, Z.R., STRAVITZ, R.T., HEUMAN, D.M., HYLEMON, P.B. & PANDAK, W.M. (1997) Quantitative estimations of the contribution of different bile acid pathways to total bile acid synthesis in the rat. *Gastroenterology*, 113, 1949–1957.

VRINS C. L. (2010) From blood to gut: direct secretion of cholesterol via transintestinal cholesterol efflux. *World J Gastroenterol*., 16, 5953–5957.

WALKER, B. R. & EDWARDS, C. R. W. (1991) 11ß-Hydroxysteroid Dehydrogenase and Enzyme-Mediated Receptor Protection: Life After Licorice. *Clinical Endocrinology,* 35, 281-289.

WALKER, B. R., CONNACHER, A. A., LINDSAY, R. M., WEBB, D. J. & EDWARDS, C. R. (1995) Carbenoxolone increases hepatic insulin sensitivity in man: a novel role for 11-oxosteroid reductase in enhancing glucocorticoid receptor activation. *J Clin Endocrinol Metab.,* 80, 3155–3159.

WALKER, B.R. (2007). Glucocorticoids and cardiovascular disease. *Eur J Endocrinol*, 157, 545-559.

WAMIL, M., BATTLE, J. H., TURBAN, S., KIPARI, T., SEGURET, D., DE SOUSA PEIXOTO R. , NELSON, Y. B., NOWAKOWSKA, D., FERENBACH D, RAMAGE, L. , CHAPMAN, K. E., HUGHES, J., DUNBAR, D. R., SECKL, J. R. & MORTON, N. M. (2011) Novel fat depot-specific mechanisms underlie resistance to visceral obesity and inflammation in 11β-hydroxysteroid dehydrogenase type 1-deficient mice. *Diabetes*, 60, 1158–1167.

WAMIL, M., & SECKL, J. R. (2007) Inhibition of 11ß-hydroxysteroid dehydrogenase type 1 as a promising therapeutic target. *Drug Discovery Today*, 12, 504-520.

WAMIL, M., ANDREW, R., CHAPMAN, K. E., STREET, J., MORTON, N. M & SECKL, J. R. (2008) 7-Oxysterols modulate glucocorticoid activity in adipocytes through competition for 11beta-hydroxysteroid dehydrogenase type. *Endocrinology*, 149, 5909–5918.

WANG, D.Q. (2007) Regulation of intestinal cholesterol absorption. *Annu Rev Physiol.*, 69, 221-48.

WANG, D.Q. & CAREY, M.C. (2002) Susceptibility to murine cholesterol gallstone formation is not affected by partial disruption of the HDL receptor SR-BI. *Biochim Biophys Acta.*, 1583, 141–150.

WANG, D.Q., COHEN, D.E. & CAREY, M.C. (2009) Biliary lipids and cholesterol gallstone disease. *J Lipid Res.*, 50, S406–S411.

WANG, N., LAN, D., CHEN, W., MATSUURA, F. & TALL, A.R. (2004) ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. *Proc Natl Acad Sci. USA,* 101, 9774–9779.

WANG, N., SILVER, D.L., COSTET, P. & TALL, A.R. (2000) Specific binding of ApoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1. *J Biol Chem.*, 275, 33053–33058.

WANG, X. & RADER, D.J. (2007) Molecular regulation of macrophage reverse cholesterol transport. *Curr Opin Cardiol.*, 22, 368–372.

WANG C. & LIAO J. K. (2012) A Mouse Model of Diet-Induced Obesity and Insulin Resistance. *Methods Mol Biol*., 821, 421–433.

WEINGÄRTNER O, WEINGÄRTNER N, SCHELLER B, LÜTJOHANN D, GRÄBER S, SCHÄFERS HJ, BÖHM M, LAUFS U. (2009) Alterations in cholesterol homeostasis are associated with coronary heart disease in patients with aortic stenosis. *Coron Artery Dis*. 6, 376-82.

WELLINGTON C. L. (2004) Cholesterol at the crossroads: Alzheimer's disease and lipid metabolism. *Clin Genet.,* 66, 1-16.

WELLINGTON, C.L., BRUNHAM, L.R., ZHOU, S., SINGARAJA, R.R., VISSCHER, H., GELFER, A., ROSS, C., JAMES, E., LIU, G., HUBER, M.T., YANG, Y.Z., PARKS, R.J., GROEN, A., FRUCHART-NAJIB, J. & HAYDEN, M.R. (2003) Alterations of plasma lipids in mice via adenoviral-mediated hepatic overexpression of human ABCA1. *J. Lipid Res.*, 44, 1470-1480.

WESTERGAARD, H. & DIETSCHY, J.M. (1976) The mechanism whereby bile acid micelles increase the rate of fatty acid and cholesterol uptake into the intestinal mucosal cell. *J Clin Invest.*, 58, 97–108.

WHITE, L.W., & RUDNEY, H. (1970) Regulation of 3-hydroxy-3-methylglutarate and mevalonate biosynthesis by rat liver homogenates. Effects of fasting, cholesterol feeding, and triton administration. *Biochemistry*, 9, 2725-2731.

WHITNEY, K. D., WATSON, M. A., GOODWIN, B., GALARDI, C. M., MAGLICH, J. M., WILSON, J. G., WILLSON, T. M., COLLINS, J. L. & KLIEWER, S. A. (2001) Liver X receptor (LXR) regulation of the LXRalpha gene in human macrophages. *J Biol Chem*., 276, 43509-15.

WILLIAMS, L. J., LYONS, V., MACLEOD, I., RAJAN, V., DARLINGTON, G. J, POLI, V., SECKL, J. R., & CHAPMAN, K. E. (2000) C/EBP regulates hepatic transcription of 11beta-hydroxysteroid dehydrogenase type 1. A novel mechanism for

cross-talk between the C/EBP and glucocorticoid signaling pathways. *J Biol Chem*., 275, 30232–30239.

WILLIAMSON, R., LEE, D., HAGAMAN, J. & MAEDA, N. (1992) Marked reduction of high density lipoprotein cholesterol in mice genetically modified to lack apolipoprotein A-I. *Proc Natl Acad Sci U S A.*, 89, 7134-7138.

WILLY P. J., UMESONO K., ONG E. S., EVANS R. M., HEYMAN R. A. & MANGELSDORF D. J. (1995) LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Dev.* 9, 1033–1045.

WILUND, K.R., YU, L., XU, F., HOBBS, H.H. & COHEN, J.C. (2004) High-level expression of ABCG5 and ABCG8 attenuates diet-induced hypercholesterolemia and atherosclerosis in Ldlr-/- mice. *J Lipid Res.*, 45, 1429–1436.

WONG, J, QUINN, C. M. & BROWN, A. J. (2006) SREBP-2 positively regulates transcription of the cholesterol efflux gene, ABCA1, by generating oxysterol ligands for LXR. *Biochem J*., 400 (3), 485-91.

WONG, J., QUINN, C. M. & BROWN, A. J. (2004) Statins inhibit synthesis of an oxysterol ligand for the liver X receptor in human macrophages with consequences for cholesterol flux. *Arterioscler. Thromb. Vasc. Biol*., 24, 2365–2371.

WORGALL, T. S., STURLEY, S. L., SEO, T., OSBORNE, T. F. & DECKELBAUM, R. J. (1998) Polyunsaturated fatty acids decrease expression of promoters with sterol regulatory elements by decreasing levels of mature sterol regulatory element-binding protein. *J Biol Chem.*, 273, 25537–25540.

WU, N., SARNA, L.K., HWANG, S.Y., ZHU, Q., WANG, P. & SIOW YL, O.K. (2013) Activation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase during high fat diet feeding. *Biochim Biophys Acta.*, 1832, 1560-1568.
WU, Z., MARTIN, K.O., JAVITT, N.B. & CHIANG, J.Y.L. (1999) Structure and functions of human oxysterol 7α-hydroxylase cDNAs and gene *CYP7B1*. *J Lipid Res.*, 40, 2195–2203.

XU, C., HE, J., JIANG, H., ZU, L., ZHAI, W., PU, S. & XU, G. (2009) Direct effect of glucocorticoids on lipolysis in adipocytes. *Mol Endocrinol.* 23, 1161–1170.

XU, J., NAKAMURA, M. T., CHO, H. P. & CLARKE, S. D. (1991) Sterol regulatory element binding protein-1 expression is suppressed by dietary polyunsaturated fatty acids. A mechanism for the coordinate suppression of lipogenic genes by polyunsaturated fats. *J Biol Chem.*, 274, 23577–23583.

YAHAGI, N., SHIMANO, H., HASTY, A. H., AMEMIYA-KUDO, M., OKAZAKI, H., TAMURA, Y., IIZUKA, Y., SHIONOIRI, F., OHASHI, K., OSUGA, J., HARADA, K., GOTODA, T., NAGAI, R., ISHIBASHI, S. & YAMADA, N. (1999) A Crucial Role of Sterol Regulatory Element-binding Protein-1 in the Regulation of Lipogenic Gene Expression by Polyunsaturated Fatty Acids. *J Biol Chem.,* 274, 35840–35844.

YAMAZAKI Y., HASHIZUME T., MORIOKA H., SADAMITSU S., IKARI A., MIWA M. & SUGATANI J. (2011) Diet-induced lipid accumulation in liver enhances ATP-binding Cassette Transporter g5/g8 expression in bile canaliculi. *Drug Metab Pharmacokinet*., 26 (5), 442-50.

YANG, L.Y., KUKSIS, A. & MYHER, J.J. (1995) Biosynthesis of chylomicron triacylglycerols by rats fed glyceryl or alkyl esters of menhaden oil fatty acids. *J Lipid Res.*, 36, 1046–1057.

YANG, Z., ZHU, X., GUO, C. & SUN K. (2009) Stimulation of 11β-HSD1 expression by IL-1β via a C/EBP binding site in human fetal lung fibroblasts. *Endocrine*, 36, 404–411.

YAO, L., HEUBI, J.E., BUCKLEY, D.D., FIERRA, H., SETCHELL, K.D., GRANHOLM, N.A., TSO, P., HUI, D.Y. & WOOLLETT, L.A. (2002) Separation of micelles and vesicles within lumenal aspirates from healthy humans: solubilization of cholesterol after a meal. *J Lipid Res.*, 43, 654–660.

YAU J. L., NOBLE J. & SECKL J. R. (2011) 11Beta-hydroxysteroid dehydrogenase type 1 deficiency prevents memory deficits with aging by switching from glucocorticoid receptor to mineralocorticoid receptor-mediated cognitive control. *J Neurosci.,* 31, 4188–4193.

YE, J., Z. GAO, J. YIN, & Q. HE. (2007) Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. *Am. J. Physiol. Endocrinol. Metab.*, 293, E1118-E1128.

YOKOYAMA C., WANG X., BRIGGS M. R., ADMON A., WU J., HUA X., GOLDSTEIN J. L. & BROWN M. S. (1993) SREBP-1, a basic-helix-loop-helix-leucine zipper protein that controls transcription of the low density lipoprotein receptor gene. *Cell*, 75 (1), 187-197.

YU L., YORK J., VON BERGMANN K., LUTJOHANN D., COHEN J. C. & HOBBS H. H. (2003) Stimulation of cholesterol excretion by the liver X receptor agonist requires ATP-binding cassette transporters G5 and G8. *J Biol Chem.*, 278 (18), 15565–15570.

YU, L., GUPTA, S., XU, F., LIVERMAN, A.D., MOSCHETTA, A., MANGELSDORF, D.J., REPA, J.J., HOBBS, H.H. & COHEN, J.C. (2005) Expression of ABCG5 and ABCG8 is required for regulation of biliary cholesterol secretion. *J Biol Chem.*, 280, 8742–8747.

YU, L., HAMMER, R.E., LI-HAWKINS, J., VON BERGMANN, K., LUTJOHANN, D., COHEN, J.C. & HOBBS, H.H. (2002b) Disruption of Abcg5 and Abcg8 in mice reveals their crucial role in biliary cholesterol secretion. *Proc Natl Acad Sci USA.*, 99, 16237–16242.

YU, L., LI-HAWKINS, J., HAMMER, R.E., BERGE, K.E., HORTON, J.D., COHEN, J.C. & HOBBS, H.H. (2002a) Overexpression of ABCG5 and ABCG8

promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol. *J Clin Invest.*, 110, 671–680.

YU, X.H., QIAN, K., JIANG, N., ZHENG, X.L., CAYABYAB, F.S. & TANG, C.K. (2014) ABCG5/ABCG8 in cholesterol excretion and atherosclerosis. *Clin Chim Acta.*, 428, 82-88.

YUAN G., WANG J. & HEGELE R. A. (2006) Heterozygous familial hypercholesterolemia: an underrecognized cause of early cardiovascular disease. *CMAJ*., 174, 1124–1129.

YUAN Y., LI P. & YE J. (2012) Lipid homeostasis and the formation of macrophage-derived foam cells in atherosclerosis. *Protein Cell*, 3 (3), 173–181.

ZELCER N., HONG C., BOYADJIAN R. & TONTONOZ P. (2009) LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. *Science*, 325, 100–104.

ZHANG Y. & MANGELSDORF D. J. (2002) LuXuRies of lipid homeostasis: the unity of nuclear hormone receptors, transcription regulation, and cholesterol sensing. *Mol Interv.,* 2 (2), 78-87.

ZHANG Y., BREEVOORT S. R., ANGDISEN J., FU M., SCHMIDT D. R., HOLMSTROM S. R., KLIEWER S. A., MANGELSDORF D. J. & SCHULMAN I. G. (2012) Liver LXRα expression is crucial for whole body cholesterol homeostasis and reverse cholesterol transport in mice. *J Clin Invest.*, 122 (5), 1688–1699.

ZHANG, S.H., REDDICK, R.L., BURKEY, B. & MAEDA, N. (1994) Diet-induced atherosclerosis in mice heterozygous and homozygous for apolipoprotein E gene disruption. *J Clin Invest*., 94, 937–*945.*

ZHANG, S.H., REDDICK, R.L., PIEDRAHITA, J.A. & MAEDA, N. (1992) Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science,* 258, 468–471.

ZHANG, W.Y., GAYNOR, P.M. & KRUTH, H.S. (1996) Apolipoprotein E Produced by Human Monocyte-derived Macrophages Mediates Cholesterol Efflux That Occurs in the Absence of Added Cholesterol Acceptors. *J Biol Chem.*, 271, 28641–28646.

ZHANG, Z. (2010). *Generation and characterisation of mice with myeloid cell-specific knockout of Hsd11b1*, Thesis (M.Sc.), School of Clinical Sciences, University of Edinburgh.

ZHAO C & DAHLMAN-WRIGHT K. (2010) Liver X receptor in cholesterol metabolism. J *Endocrinol.*, 204 (3), 233-40.

ZHAO, .SP., YU, B.L., XIE, X.Z., DONG, S.Z. & DONG, J. (2008) Dual effects of oxidized low-density lipoprotein on LXR-ABCA1-apoA-I pathway in 3T3-L1 cells. *Int J Cardiol.*, 128, 42-47.

ZHU, R., OU, Z., RUAN, X., & GONG, J. (2012) Role of liver X receptors in cholesterol efflux and inflammatory signaling (Review). *Mol Med Rep.*, 5 (4), 895-900.

ZIBOH, V.A., MATSCHINER, J.T., DOISY, E.A. Jr, HSIA, S.L., ELLIOTT, W.H., THAYER, S.A. & DOISY, E.A. (1961) Bile acids. XIV. Metabolism of chenodeoxycholic acid-24-C-14 in surgically jaundiced mice. *J Biol Chem.*, 236, 387–390.

ZIEDEN, B., KAMINSKAS, A., KRISTENSON, M., KUCINSKIENE, Z., VESSBY, B., OLSSON, A.G., & DICZFALUSY, U. (1999) Increased plasma 7ß-hydroxycholesterol concentrations in a population with a high risk for cardiovascular disease. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 19, 967-971.

ZOU X., PELLICORO A., AUCOTT R., RAMACHANDRAN P., CLARKSON M., WEBSTER S.P., IREDALE J.P., WALKER B.R. & MICHAILIDOU Z. (2013). 11β-HSD1 deficiency increases susceptibility to liver fibrosis by activating hepatic stellate cells. Society for Endocrinology BES 2013, Harrogate, UK. *Endocrine Abstracts* (2013), 31, P315.

ZOU, X., PELLICORO, A., AUCOTT, R., RAMACHANDRAN, P., CLARKSON, M., WEBSTER, S. P., IREDALE, J. P., WALKER, B. R. & MICHAILIDOU, Z. (2013) 11βHSD1 deficiency increases susceptibility to liver fibrosis by activating hepatic stellate cells. *Endocrine Abstracts* 31, P315

ZOU, Y., LI, J., LU, C., WANG, J., GE, J., HUANG, Y., ZHANG, L. & WANG, Y. (2006) High-fat emulsion-induced rat model of nonalcoholic steatohepatitis. *Life Sci.*, 79, 1100–1107.

Appendix

Awards and Abstracts

Awards

2013	Travel Award by Society for Endocrinology, for the BES 2013 Meeting in Harrogate, UK (£500)
2013	Travel Award by Society for Endocrinology, for the ENDO 2013 Meeting in San Francisco, USA (£850)
2012	Career Development Workshop Award, Society for Endocrinology

Abstracts

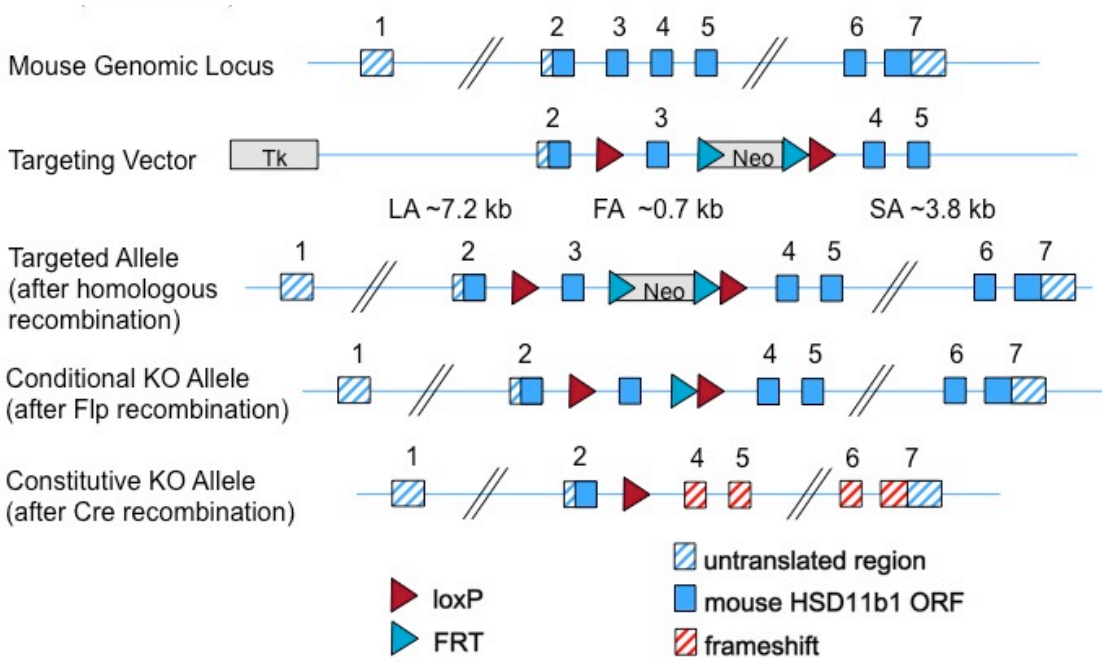
Manwani K., Man T.Y., Kenyon C., Andrew R., Chapman K.E., Seckl J.R. **"Hepatic 11β-hydroxysteroid dehydrogenase type 1 and atherosclerosis risk."** *Endocrine Society Annual Meeting and Expo, San Francisco, USA, 2013, poster abstract*

Manwani K., Man T.Y., Kenyon C., Andrew R., Chapman K.E., Seckl J.R. **"The role of hepatic 11β-hydroxysteroid dehydrogenase type 1 in cholesterol homeostasis."** *British Endocrine Society Meeting, Harrogate, UK, poster abstract*; *Endocrine Abstracts (2013) 31, P181.*

Manwani K., Man T.Y., Kenyon C., Andrew R., Chapman K.E., Seckl J.R. **"Hepatic 11β-hydroxysteroid dehydrogenase type 1 and its role in cholesterol metabolism."** *Scottish Society for Experimental Medicine Meeting, Edinburgh, UK, 2013, poster abstract*

Manwani K., Man T.Y., Seckl J.R., Chapman K.E. **"Hepatic 11β-HSD1 and atherosclerosis risk."** *BHF Centre for Cardiovascular Science Symposium Day, Edinburgh, UK, 2012, poster abstract*

Targeting strategy for generation of *Hsd11b1* conditional allele by TaconicArtemis



Suppliers' addresses

Abcam	330 Cambridge Science Park, Cambridge, CB4 0FL, UK
Agilent Technologies	610 Wharfedale Road, Wokingham, RG41 5TP, UK
Amersham Pharmacia Biotech	Amersham Place, Little Chalfont, HP7 9NA, UK
Beckman Coulter	Oakley Court, Kingsmead Business Park, London Road, High Wycombe, HP11 1JU, UK
Bio-Rad Laboratories Ltd.	Maxted Road, Hemel Hemstead, HP2 7DX, UK
Biotium, Inc.	Munro House, Trafalgar Way, Cambridge, CB23 8SQ, UK
Denver	Denver House, Sovereign Way, Trafalgar Business Park, Downham Market, Norfolk PE38 9SW, UK
Eppendorf	Arlington Business Park, Stevenage, SG1 2FP, UK
Fine Science Tools Inc.	Vangerowster 14, DE-69115, Heidelberg, Germany
GE Healthcare	Amersham Place, Little Chalfont, HP7 9NA, UK
Hook & Tucker Instruments	Vulcan Way New Addington, Croydon, Surrey, CR0 9UG, UK
IKA	Janke&Kunkel-Str. 10, 79219 Staufen, Germany
Invitrogen	3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, UK
Jenway	Beacon Road, Stone, Staffordshire, ST15 0SA, UK
Leica	Davy Avenue Knowlhill, Milton Keynes, MK5 8LB, UK
LI-COR Biosciences	St. John's Innovation Centre, Cowley Road, Cambridge CB4 0WS, UK
Lonza	228 Bath Road, Slogh, Berkshire, SL1 4DX, UK
Mettler Toledo	64 Boston Road, Beaumont, Leicester, LE4 1AW, UK
Millipore	Croxley Green Business Park, Watford, WD18 8YH, UK

Molecular Devices	60-665 Eskdale Road, Wokingham, RG41 5TS, UK
NanoDrop Technologies	3411 Silverside Rd, Wilmington, DE 19810, USA
New England Biolabs	75/77 Knowl Piece, wilbury Way, Hitchin, SG4 0TY, UK
Promega	Southampton Science Park, Southampton, SO16 7NS, UK
Qiagen	Fleming Way, Crawley, West Sussex, RH10 9NQ, UK
Roche Applied Science	Charles Avenue, Burgess Hill, RH15 9RY, UK
StarLab	Tanners Drive, Blakelands, Milton Keynes, MK14 5NA, UK
Stuart Equipment	(Bibby Scientific Ltd.) Beacon Road, Stone, Staffordshire, ST15 0SA, UK
Swann-Morton Ltd.	Owlerton Green, Sheffield, S6 2BJ, UK
TAAB Laboratories	3 Minerva House, Calleva Park, Aldermaston, RG7 8NA, UK
Techne	(Bibby Scientific Ltd.) Beacon Road, Stone, Staffordshire, ST15 0SA, UK
Thermo Fisher Scientific	Stafford House, Boundary Way, Hemel Hempstead, HP2 7GE, UK
UVItec	Unit 36, St. John's Innovation Centre, Cowley St., Cambridge, CB4 0WS, UK
VWR International Ltd.	Hunter Boulevard, Magna Park, Lutterworth, LE17 4XN, UK
Whatman	Part of GE Healthcare at present
Zeiss	509 Coldhams Lane, Cambridge, CB1 3JS, UK